CHARACTERIZATION OF AUXILIARY SUBUNITS FOR GLR-1 AMPA RECEPTORS IN *CAENORHABDITIS ELEGANS*

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

In vertebrates the vast majority of the excitatory neurotransmission is mediated by glutamate receptors. Are they stand-alone molecules? How is their function regulated to ensure proper signaling for normal physiology and behavior? To address these questions I have undertaken combined molecular, genetic, electrophysiological and behavioral analyses centered on GLR-1 AMPA-class ionotropic glutamate receptor (iGluR), one of the major components of glutamate receptor signaling in simple nematode *Caenorhabditis elegans*. This dissertation describes my identification and characterization of two families of GLR-1 auxiliary subunits.

By taking both reverse and forward genetic approaches I isolated STG-1 and STG-2, two TARPs (Transmembrane AMPA receptor Regulatory Proteins), as the essential set of obligate accessory proteins for worm GLR-1 AMPA receptors. I showed that the elimination of both STG-1 and STG-2 causes disruption of AMPAR-mediated synaptic currents and worm behavior despite normal surface expression and clustering of the receptors. By reconstituting AMPAR function in heterologous systems, I demonstrated that both STG-1 and STG-2 can functionally substitute for vertebrate TARPs to modify receptor function, indicative of their evolutionarily conserved role. In

addition, I also showed that STG-1 and STG-2 differentially alter the kinetics of *C. elegans* GLR-1 and vertebrate GluR1 (GluA1) receptors.

By taking a forward genetic approach in a sensitized genetic background, I identified a CUB-domain protein – SOL-2 that contributes to the function of GLR-1 AMPARs. I showed that the deletion of SOL-2 significantly reduces the AMPAR-mediated currents and elicits behavioral changes. I also demonstrated that SOL-2 is required to bind to and stabilize the extracellular part of SOL-1, a previously identified obligate auxiliary subunit of GLR-1, and this binding complex co-localizes with GLR-1. These data suggest that a protein complex containing CUB-domains has a scaffolding role and contributes to the function of AMPARs.

Together, I identified two families of accessory proteins – TARPs and CUB-domain proteins that are required for the function of AMPARs, supporting the hypothesis that glutamate receptors exert their function by forming signaling complexes with auxiliary subunits instead of working alone.

To my family

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CHAPTER 1

INTRODUCTION

Synapses and Receptors

The nervous system enables us to perceive and react to the environment, and also allows for our mental activities, such as thinking, learning and memory. Its control and regulation of our behavior are based upon its communicative function as it sends and receives nerve impulses throughout our body. As a key step in neural activities, the transmission of impulses occurs through synapses, the highly specialized contacting points between two nerve cells (neurons). Through synapses, nerve cells signal to each other and to non-neuronal cells, such as muscle cells or gland cells. In our body trillions of such synapses integrate billions of neurons into organized neural networks (neural circuits) thereby making it possible to regulate complex behavioral and cognitive activities.

There are two types of synapses in the nervous system, electrically coupled gap junctions and chemical synapses. Gap junctions are tightly coupled, bi-directional pores where electrical signals pass through, directly and fast (Bennett 2000). Chemical synapses are what we commonly refer to as "synapses." Compared to gap junctions, cells across a chemical synapse are not tightly coupled, but separated by a small place called synaptic cleft. Moreover, the passage of signals at chemical synapses is unidirectional from presynaptic cell to postsynaptic cell, highlighting the structural and functional asymmetries between presynaptic and postsynaptic terminals. The presynaptic terminal contains synaptic vesicles that are gathered and docked near the plasma membrane. On the opposite side of the synaptic cleft, neurotransmitter receptors reside on the surface of the postsynaptic terminal. Underneath lies the postsynaptic density (PSD), an electron dense

region composed of many interlinked protein complexes. During a typical excitatory synaptic neurotransmission process, nerve impulses undergo an electrical-chemical-electrical change. The electrical activation (action potential) of the presynaptic cell causes synaptic vesicles to fuse with plasma membrane and therefore release their content, the neurotransmitters that are enclosed within synaptic vesicles. The neurotransmitters then diffuse across the synaptic cleft and bind to receptors on postsynaptic membrane, causing the activation of the receptors and the opening of ion channels. Cations then influx and depolarize (activate) the postsynaptic cell (or activate signaling cascades). The depolarization of the cell elicits the formation of action potentials, thereby starting a new cycle of electrical-chemical signal conversion and resuming the process of neurotransmission (Figure 1.1).

Neurotransmitters are typically small chemicals, such as acetylcholine, glutamate, γ-aminobutyric acid (GABA), dopamine and serotonin. They activate two broad categories of receptors based on their structural and functional differences. Metabotropic receptors do not form ion channel pores but the binding of neurotransmitters induces intracellular signaling pathways that eventually activate indirectly linked ion channels. This process takes seconds or minutes. Alternatively, neurotransmitters activate ligand-gated ionotropic receptors by directly gating ion channels formed by receptors upon binding. This occurs in milliseconds or less.

The activation of a receptor by its neurotransmitter results in either an “excitatory”

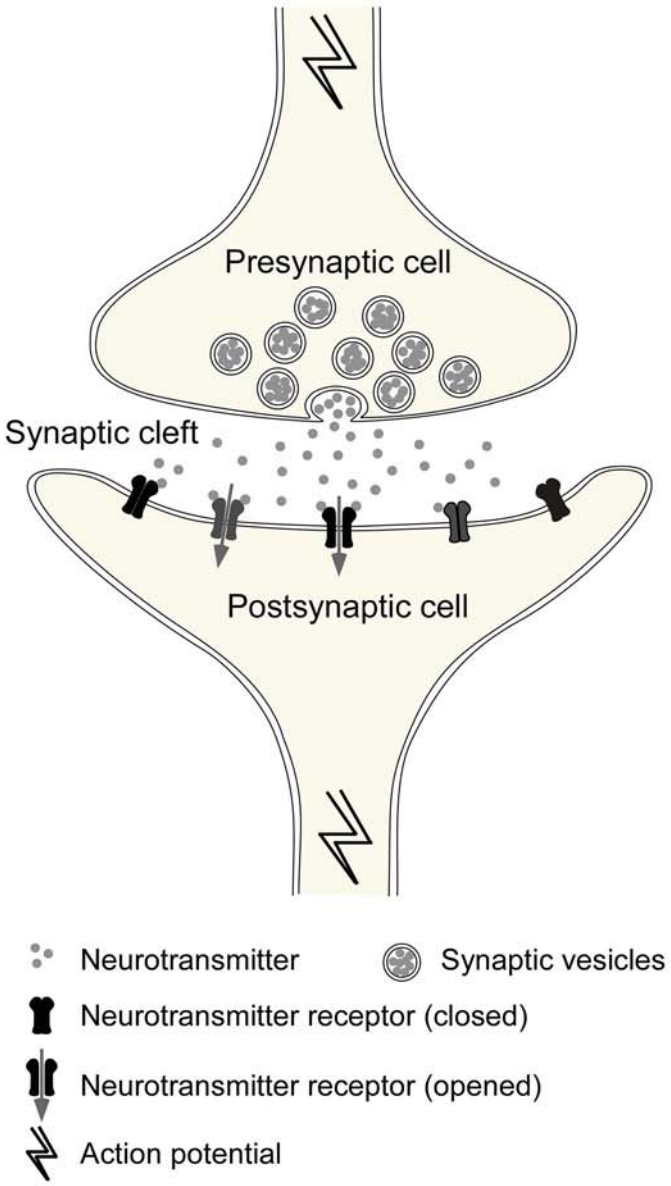

Figure 1.1 The neurotransmission at chemical synapses. The presynaptic terminal and postsynaptic terminal are separated by a synaptic cleft. In the presynaptic cell, the neurotransmitter is stored in synaptic vesicles. Upon the reaching of electrical signal (action potential), the synaptic vesicles fuse with plasma membrane and release neurotransmitters, which diffuse across the synaptic cleft and bind to receptors on the postsynaptic membrane. The binding causes the activation of receptors and the opening of ion channels. Ion flows in and the cell is depolarized, inducing the formation of action potential.

or “inhibitory” neurotransmission depending on the properties of the neurotransmitter and its receptor. Excitatory neurotransmission depolarizes the postsynaptic cell and therefore increases the probabilities to fire an action potential (as shown in Figure 1.1) whereas inhibitory transmission hyperpolarizes the cell and reduces its firing capability.

In the vertebrate central nervous system (CNS), the majority of the excitatory neurotransmission is mediated through the ionotropic glutamate receptors (iGluRs). These receptors also play fundamental roles in synaptic plasticity, the underlying molecular mechanism of learning and memory (Asztely et al. 1996; Riedel et al. 2003). Due to their pivotal roles in excitatory neurotransmission, the misregulation or disruption of the normal signaling via iGluRs is implicated in a wide range of neuropathological and physiological disorders and diseases, such as epilepsy and brain damage, Parkinson’s and Alzheimer’s diseases, Huntington’s disease, amyotrophic lateral sclerosis, and therefore iGluRs become important drug targets for therapeutic purposes (Bleich et al. 2003).

Structure and Function of Ionotropic Glutamate Receptor Subunits

The mammalian ionotropic glutamate receptors are encoded by 18 genes and can be divided into three major subtypes based on their pharmacological properties and associated physiological functions (Figure 1.2, according to the new nomenclature developed by NC-IUPHAR (Collingridge et al. 2009)). NMDA receptors are the first subtype and can be selectively activated by the agonist NMDA (N-methyl D-aspartate).

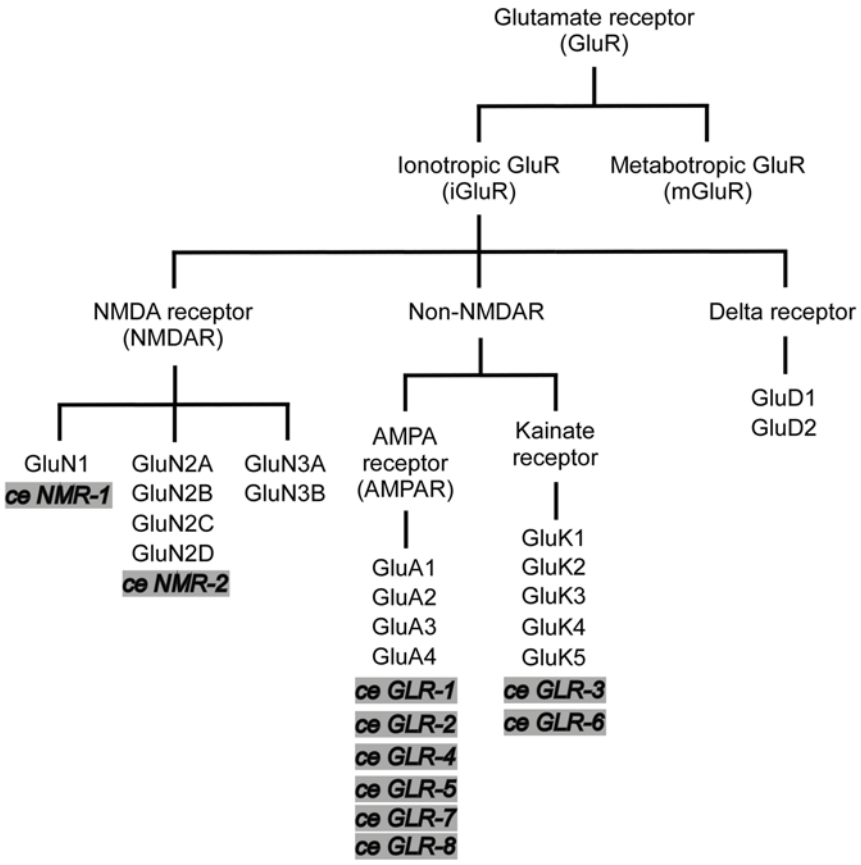

Figure 1.2 The glutamate receptor subunits. Glutamate receptors are divided into ionotropic and metabotropic receptors. The ionotropic glutamate receptors are classified as 3 subtypes, the NMDA, non-NMDA and delta receptors. Non-NMDA class comprises AMPA-type and kainate-type receptors. The nematode *C. elegans* has iGluR homologues that fall into almost each subtype, shown by italicized shaded blocks.

NMDARs are heteromers containing both GluN1 and GluN2 (GluN2A-D) and sometimes GluN3 (GluN3A and GluN3B). The second subtype are the non-NMDA receptors, including AMPA receptors and kainate receptors that are preferentially sensitive to AMPA (amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate respectively (Collingridge et al. 1989; Monaghan et al. 1989). AMPARs are assembled as homo- or hetero-tetramers that contain GluA1-4 subunits. Kainate receptors are also tetrameric channels assembled by GluK1-5. The third subtype are the orphan-type delta receptors, which show some sequence homology to AMPA/kainate receptors but appear not to form functional channels (Lomeli et al. 1993). GluD1 and GluD2 belong to this category.

The iGluRs are highly conserved across the animal kingdom. The nematode *C. elegans* has at least 10 homologues in its genome (Brockie et al. 2001). Among them NMR-1 and NMR-2 are most similar to NMDA subunits and GLR-1 to GLR-8 show homology to non-NMDA subtypes. GLR-3 and GLR-6 bear similarities to kainate subtypes whereas the rest could be AMPA-type subunits (Figure 1.2).

All the iGluR subunits share the same membrane topology and same modular domain organization (Figure 1.3). They are multipass membrane spanning proteins. Each subunit has an extracellular amino terminus and an intracellular carboxyl terminus. There are two extracellular domains. The first one is the amino-terminal domain (ATD), which is believed to be involved in protein-protein interactions, such as, the subunit oligomerization and perhaps the trafficking of the receptors (Leuschner et al. 1999; Ayalon et al. 2001; Qiu et al. 2009). The other one is the ligand binding domain (LBD), which is

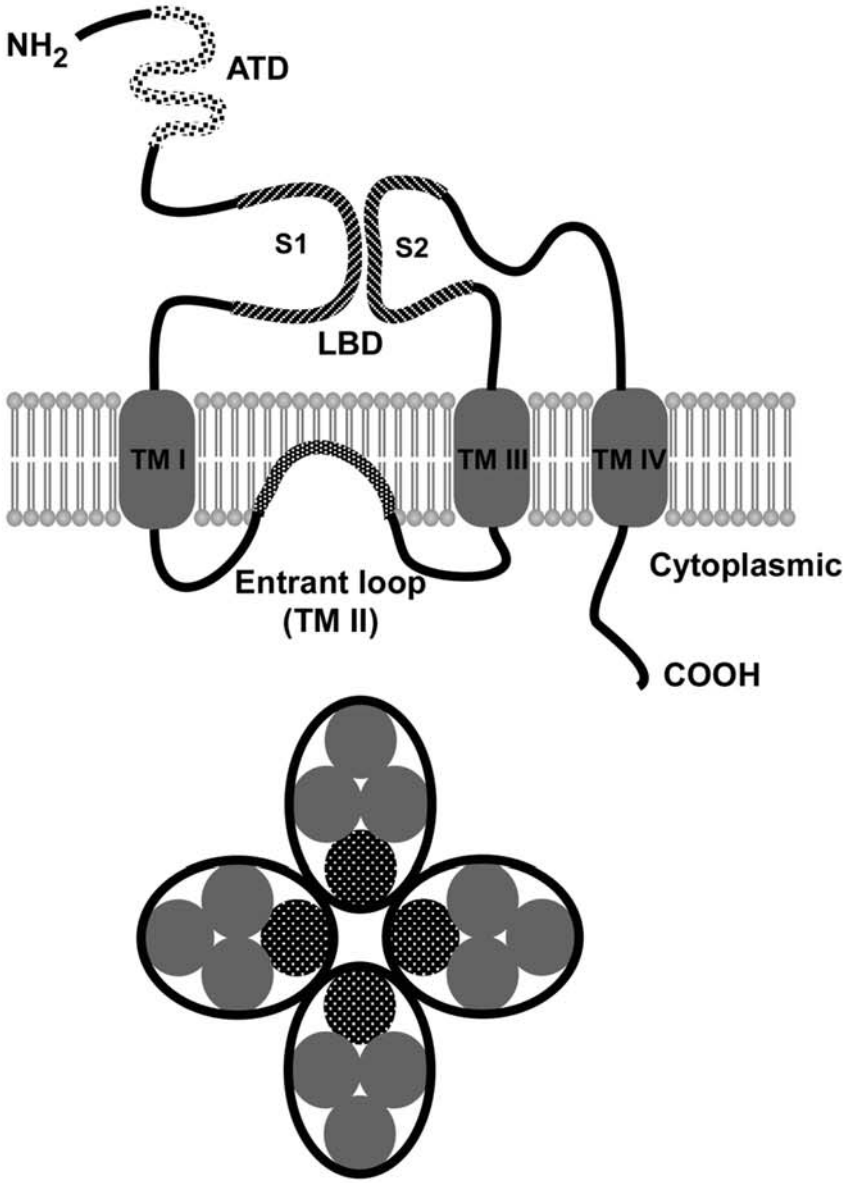

Figure 1.3 Transmembrane topology and subunit stoichiometry of ionotropic glutamate receptor. (Top) Subunits of iGluRs are composed of an amino-terminal domain (ATD), followed by the S1 domain, the first transmembrane domain (TM I), a re-entrant loop that lines the channel pore (TM II), and two other transmembrane domains (TM III and IV) separated by the S2 loop. The S1 and S2 form the ligand binding domain (LBD). (Bottom) A functional iGluR channel is composed of four subunits and the pore of the channel is lined up by the re-entrant loops from all four subunits.

formed by the intertwined S1 and S2 domains. S1 domain follows the amino-terminal domain (ATD), whereas S2 domain lies between the last two transmembrane domains (TM III and IV). LBD is similar to the bacterial glutamine binding protein (QBP) (Hsiao et al. 1996; Armstrong et al. 1998) and is involved in agonist binding and receptor kinetics (Stern-Bach et al. 1994; Stern-Bach et al. 1998). Crystallographic studies have revealed that S1 and S2 domains form two lobes separated by an agonist binding cleft (Paas 1998). The ion channel domain comprises three transmembrane domains (TM I, III and IV) and a re-entrant loop (TM II) that lines the inner pore. The intracellular carboxyl terminal domain has variable length depending on subunit subtype. The C-terminal tail is a target of post-translational modifications. For example, the phosphorylation of the intracellular tail of AMPARs is reported to be associated with activity-dependent receptor function (Dingledine et al. 1999; Boehm et al. 2005). The C-terminus is also involved in protein-protein interactions, most notably through the PDZ binding motifs of AMPARs that interacts with PDZ domains of postsynaptic density proteins, thereby regulating synaptic localization and forming supramolecular signaling complexes (Sheng et al. 1997).

It is widely accepted that each glutamate receptor subunit co-assembles with other subunits from the same subtype group and functional tetrameric iGluRs are dimers of dimers (Ayalon et al. 2001; Mayer et al. 2001; Tichelaar et al. 2004). Recent crystallographic studies provide further support for the “dimers of dimers” stoichiometry of iGluRs. The 3.6Å resolution reveals an overall 2-fold symmetry of the rat tetrameric GluA2 receptors. The extracellular domains and the ion channel domain exhibit a 2-fold

and 4-fold symmetry respectively (Sobolevsky et al. 2009). However, deciphering the exact subunit composition of functional iGluR channels *in vivo* is still a daunting task considering that alternative splicing and RNA editing of receptor subunit would add extra diversity. Recently, Lu et al. quantitatively determined the AMPAR subunit composition in a part of the CNS using single-cell genetic approach combined with electrophysiology (Lu et al. 2009).

Upon binding glutamate, non-NMDA receptors rapidly open an integral pore allowing the entry of monovalent cations K^+ and Na^+, and sometimes, the divalent cation Ca^{2+} when the GluA2 subunit is absent from the receptors. The permeability of Ca^{2+} is determined by a key neutral glutamine (Q) residue in the re-entrant loop (TMII) domain. In GluA2 this residue is replaced with a positively charged arginine (R) by RNA editing, which blocks the passage of Ca^{2+} (Sommer et al. 1991; Burnashev et al. 1992).

Studies from Lurcher mice have revealed another key residue in TMIII that determines the opening state of δ2 (GluD2) receptor (Zuo et al. 1997). The replacement of a highly conserved alanine (A) with threonine (T) in the SYTANLAAF motif (Figure 1.4) causes constitutively open (leaky) channels and the apoptotic death of Purkinje cells in the cerebellum, leading to gait abnormality from which the name "*lurcher*" is derived. In the line with a rapid opening of the pore, non-NMDA receptors also show fast desensitization in the continued presence of glutamate, consistent with its physiological role – the mediation of fast excitatory neurotransmission. Compared to non-NMDA receptors, NMDA receptors show marked differences in kinetics and ion permeability. First, both

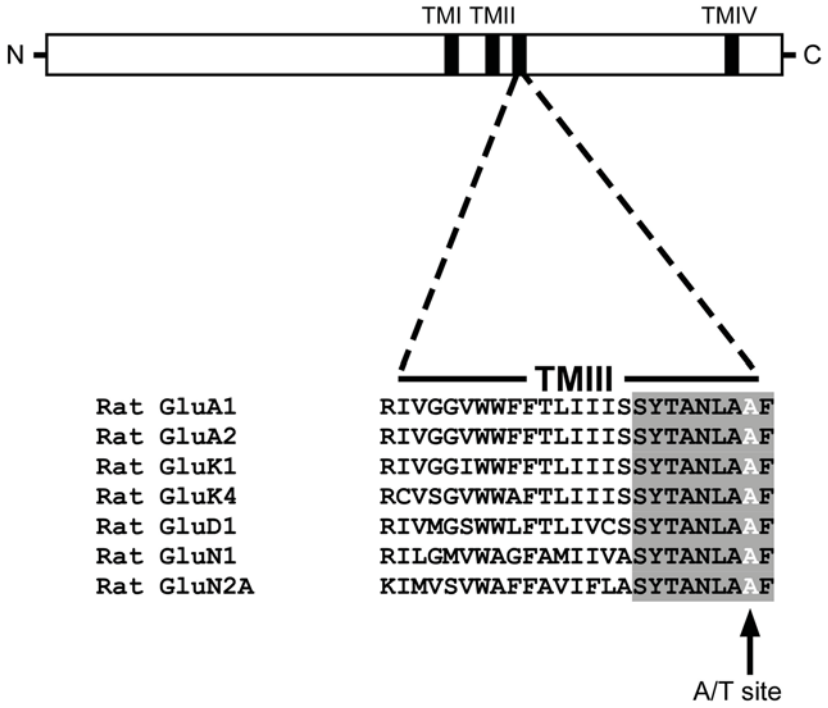

Figure 1.4 A highly conserved alanine (A) in the SYTANLAAF motif of TM III. A/T site marks the dominant mutation that was introduced into the transgenic worms used in genetic screens in Chapter 2 and 3.

glutamate and its coagonist, glycine or D-serine, must bind to open the pore of the channel (Johnson et al. 1987; Benveniste et al. 1991; Mothet et al. 2000). Most importantly, NMDA receptors are highly permeable to Ca^{2+} but are blocked by extracellular Mg^{2+} and their activation requires depolarization of the postsynaptic cell to remove the voltage-dependent Mg^{2+} blockade from the pore (Mayer et al. 1984; Nowak et al. 1984; Jahr et al. 1990). Thus, in order to activate NMDA receptors the binding of agonists and the depolarization of membrane must coincide in time, suggestive of a potential role as a molecular coincidence detector for glutamate release and depolarization, and therefore a key in understanding long term potentiation and depression (Bliss et al. 1993). This unique feature inspires numerous studies aimed at the elucidation of the molecular and cellular mechanisms of learning and memory. Additionally, both the activation and desensitization of NMDA receptors are much slower compared to those of non-NMDA receptors (Trussell et al. 1989). Therefore, NMDA receptors might mediate the slow and long-lasting components of the excitatory currents (Lester et al. 1990).

Regulation of Ionotropic Glutamate Receptors

Before nascent iGluRs become activated in response to their agonists under physiological conditions, they need to be assembled in combinations of subunits, trafficked to specific cytosolic compartments, inserted into postsynaptic membrane and clustered by associating with postsynaptic proteins. On each of these steps the function of iGluRs must be properly regulated. Moreover, additional fine-tuning of iGluR function comes from

activity-dependent modifications and modulations.

The intrinsic subunit properties and the composition of subunits are the major determinants of iGluR function. Introduction of posttranscriptional and posttranslational modifications to iGluR subunits can significantly change iGluR function and therefore, alter synaptic transmission. Alternative splicing and RNA editing are the major forms of RNA modifications that regulate iGluRs. Alternative splicing generates “flip” and “flop” forms of AMPARs, with nine different residues in S2 loop preceding TM III (Sommer et al. 1990). These splicing variants show distinct channel kinetics and their expression is developmentally regulated (Monyer et al. 1991). Numerous other splicing variants of iGluRs are also reported (Dingledine et al. 1999). In addition to alternative splicing, RNA editing leads to more molecular diversity. Q/R editing dramatically changes Ca^{2+} permeability, channel conductance and other channel properties of GluR2 (GluA2)-containing AMPARs (Hume et al. 1991; Swanson et al. 1996). Q/R editing also takes place in GluR5 (GluK1) and GluR6 (GluK2). Furthermore, R/G editing of GluR2-4 (GluA2-4) is reported to accelerate recovery from desensitization (Lomeli et al. 1994). RNA editing is also found in GluR5-7 (GluK1-3) kainate receptors (Kohler et al. 1993; Nutt et al. 1994).

In addition to RNA-processing events, posttranslational modifications are also important mechanisms for regulating iGluR function. Phosphokinases, such as PKA, PKC, CaMKII and other unidentified kinases potentiate glutamate receptor function and thereby synaptic transmission whereas phosphotase calcineurin removes phosphate residues and

inhibits receptor function (Dingledine et al. 1999). The phosphorylation and dephosphorylation events occur on serine/threonine, or tyrosine residues in some cases. In addition, glycosylation also regulates several properties of glutamate receptor channels (Dingledine et al. 1999).

Protein-protein interaction is another important way to regulate iGluR function. Multiple families of proteins are involved in the proper localization and function of iGluRs, among which PDZ (PSD-95, Discs large, ZO-1) domain containing protein have been intensively characterized. PDZ domains are comprised of approximately 90 amino acids that bind to the C-terminal S/T-X-V/I or F/Y-X-V motif of target proteins (Songyang et al. 1997). PDZ domain proteins associate with other postsynaptic proteins including iGluRs via their PDZ domains and other structural domains, thereby forming multiple-domain scaffolds and multiple-protein complexes, localizing synaptic proteins and mediating signaling cascades (Kim et al. 2004). At glutamatergic synapses, PDZ domain proteins, including PICKI (Protein Interacting with C Kinase I), PSD-95(Post-Synaptic Density-95) family proteins, GRIP (Glutamate Receptor Interacting Protein) and ABP (AMPAR Binding Protein), interact directly with the conserved C-terminal PDZ domain-binding motifs of iGluRs.

A number of PSD-95 family proteins, including PSD-95 (also known as SAP90, Synapse-Associated Protein 90), PSD-93 and SAP102, directly bind to the NR2 (GluN2) subunit of NMDA receptors and regulate their localization and function (Kornau et al. 1995; Sheng et al. 1997; Kim et al. 2004). The direct association of PSD-95 family proteins

and NMDAR C-termini is found in several *in vitro* and *in vivo* experiments (Kornau et al. 1995; Lau et al. 1996; Muller et al. 1996; Niethammer et al. 1996). In heterologous cells PSD-95 can cluster NR2 (GluN2) subunits (Kim et al. 1995). In mutant mice lacking PSD-95 impaired learning was observed, similar to NMDAR knockout animals (Migaud et al. 1998).The C-terminus of NMDARs is also essential for localization and function (Kim et al. 1995; Mori et al. 1998; Steigerwald et al. 2000).

In addition to PDZ domain proteins, iGluRs bind to a variety of other cytoskeletal, scaffolding, adaptor, anchoring, structural, and signaling proteins mainly via their intracellular C-terminal tails. In particular, several receptor subunits can bind directly to signaling proteins. For example, NMDA receptor subunits bind to Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II (Gardoni et al. 1998; Strack et al. 1998; Leonard et al. 1999; Leonard et al. 2002).

Together, the interactions between iGluR subunits and regulatory proteins allow for fine-tuning of receptor function with temporal and spatial specificity.

Auxiliary Subunits for iGluRs

In addition to PDZ scaffolding, signaling and other regulatory proteins in postsynaptic density, specific families of auxiliary integral proteins have been found to modulate the expression and function of iGluRs. These proteins include, but are not limited to, TARPs, CUB-domain containing Neto proteins and perhaps cornichon-like proteins.

The first family of auxiliary proteins for iGluRs is TARP (Transmembrane AMPA Receptor Regulatory Protein). The archetypal TARP, stargazin, was isolated from *stargazer* (*stg*) mutant mice that are ataxic and epileptic (Letts et al. 1998). Stargazin encodes a 38kD protein and shares homology with nonpore forming, skeletal muscle L-type Ca^{2+} channel γ-1 subunit, and therefore was named γ-2. It was reported to affect Ca^{2+} channel currents *in vitro* and therefore was thought to be part of Ca^{2+} channel (Letts et al. 1998). Later studies, however, identified stargazin as an essential regulator of AMPA receptor synaptic expression (Chen et al. 2000). The calcium currents and NMDA-mediated currents are normal in *stg/stg* cerebellar granule cells but AMPA receptor currents are impaired, indicating that stargazin's function is specific to AMPARs (Chen et al. 1999; Hashimoto et al. 1999; Chen et al. 2000). Also, stargazin associates with both AMPA receptors and PSD-95. Wild-type stargazin but not the C-terminal truncated version (in which PDZ domain binding motif is deleted), rescues the synaptic AMPAR current, indicating that its PDZ domain binding motif is essential for function. However, the truncated stargazin rescues extrasynaptic AMPAR responses. Based on these data, stargazin is proposed to have two distinct roles. First, stargazin delivers AMPARs to the membrane surface, a role that does not require its PDZ domain binding motif. Second, stargazin moves AMPARs to correct synaptic zones, and this step is dependent on the binding of its C-terminus with postsynaptic density proteins (Chen et al. 2000).

TARPs are four-pass transmembrane proteins with cytosolic N and C termini. Other canonical class I TARP family members comprise γ-3, γ-4 and γ-8 (Tomita 2010).

They are expressed differentially in all brain regions and also mediate surface expression of AMPARs (Tomita et al. 2003). Stargazin is mainly expressed in cortex, midbrain, hippocampus, pons, cerebellum and thalamus, whereas γ-3, γ-4 and γ-8 are expressed in cerebral cortex, olfactory bulb and hippocampus, respectively (Letts et al. 1998; Sharp et al. 2001; Moss et al. 2003; Tomita et al. 2003). Atypical isoforms γ-5 and γ-7 (class II) also act as TARPs and are expressed in many brain regions but display distinct physiological features compared to other TARP isoforms (Kato et al. 2007; Soto et al. 2009; Tomita 2010).

In addition to targeting AMPARs to synapses, TARPs can modify AMPAR channel properties in several ways: slowing desensitization and deactivation, increasing open probabilities, modulating gating, reducing polyamine block, enhancing kainate efficacy and changing agonist and antagonist pharmacology of AMPARs (Priel et al. 2005; Tomita et al. 2005; Turetsky et al. 2005; Tomita et al. 2006; Kott et al. 2007; Menuz et al. 2007; Milstein et al. 2007; Soto et al. 2007). Class I isoforms exhibit overall redundant but slightly different modulatory effects, whereas class II isoforms appear to have more specialized and distinct functions. The isoform γ-7 resembles canonical TARPs in some functions such as receptor trafficking and modifying kinetics, but γ-7 differentially modulates the pharmacology of AMPARs for glutamate and kainate, and only partially restores functional AMPARs on *stargazer* cerebellar granule cells (Kato et al. 2007). Furthermore, γ-7 binds to an mRNA-binding protein hnRNP A2 (heterogeneous nuclear ribonucleoprotein A2) and controls $Ca_V2.2$ mRNA stability (Ferron et al. 2008). γ-5 was

initially thought not to be a TARP since it cannot increase AMPAR currents in cerebellar granule cells or heterologous cells (Kato et al. 2007) and does not regulate receptor trafficking or gating (Tomita et al. 2003). However, γ-5 still regulates AMPARs, but in specific combinations and in different mechanisms. γ-5 specifically modulates the function of GluR2-containing AMPARs by increasing peak currents and decreasing steady-state currents, increasing deactivation and desensitization rates, and decreasing glutamate potency (Kato et al. 2008). Also, γ-5 was found to selectively regulate long-form calcium-permeable AMPA receptors in Bergmann glia (Soto et al. 2009).

TARPs have evolutionarily conserved roles. Functional homologues have been identified from *C. elegans*, *Drosophila* and *Apis mellifera* and they all act as modulators of AMPAR functional properties (Walker et al. 2006; Walker et al. 2006; Wang et al. 2008). In particular, two worm TARP homologues STG-1 and STG-2 are essential for AMPAR mediated currents and related function (Chapter 2) (Wang et al. 2008).

TARPs are the first known family of auxiliary subunits for iGluR ion channels. Since the discovery of TARPs it has been widely accepted that iGluRs are not "stand-alone" molecules but instead require auxiliary subunits for their function. This has promoted the search and discovery of other iGluR auxiliary subunits.

In 2009 a novel family of cornichon-like proteins was reported to be AMPAR auxiliary subunits (Schwenk et al. 2009). Cornichon proteins are three pass transmembrane proteins with an intracellular N-terminus and an extracellular C-terminus. In *Drosophila*, cornichon is required for establishing both anterior-posterior and dorso-ventral polarity in

embryos by acting as cargo receptor for ER export of the TGFα-like growth factor Gurken (Roth et al. 1995; Bokel et al. 2006). In yeast, the cornichon-related protein Erv14 is required for the ER export of most transmembrane proteins (Castillon et al. 2009). Similarly, the mammalian cornichon homologues also facilitate the transport and secretion of cargo from the ER (Castro et al. 2007; Hoshino et al. 2007).

Schwenk et al identified cornichon-like proteins, CNIH-2 and CNIH-3, as tightly associated AMPAR-interacting proteins from rat brain using proteomic approaches (Schwenk et al. 2009). These proteins, like TARPs, bind robustly with AMPARs and are widely expressed in many brain regions. They also regulate the trafficking and function of AMPARs. CNIH-2 and CNIH-3 increase the surface expression of AMPARs, consistent with the conserved function of their homologues and showing a similar role to TARPs in trafficking of AMPARs. Furthermore, cornichon-like proteins and TARPs both increase the total charge transfer through AMPARs by slowing the desensitization and deactivation kinetics of the channels.

Additional features distinguish TARPs from Cornichons. First of all, their membrane topology and structural domains are different, suggestive of different functions. For example, TARPs have C-terminal PDZ-binding motifs that bind to PDZ proteins, while CNIH-2 and CNIH-3 do not. Second, CNIH-2 and CNIH-3 are absent in cerebellar granule cells where stargazin is expressed and required for the trafficking and function of AMPARs. Third, it appears that more AMPARs associate with CNIH-2 and CNIH-3(70%) than TARPs (30%), implicating the presence of distinct CNIH- and TARP-associated AMPAR

pools during trafficking and on the membrane surface, and the potential functional differences between these pools. Moreover, CNIH-2 and CNIH-3 have a bigger effect on slowing desensitization and deactivation of AMPAR channel kinetics than TARPs (Jackson et al. 2009; Schwenk et al. 2009; Tigaret et al. 2009).

However, further comparative studies showed that although cornichon-like proteins mimic some of the functions of TARPs in heterologous cells, they are not detected on the surface of the neurons and they fail to change the kinetics of endogenous AMPARs, more consistent with their classical roles as endoplasmic reticulum (ER) chaperons (Shi et al. 2010). Thus, CNIHs are probably not bona-fide AMPAR auxiliary subunits, but instead, only temporarily associated with AMPARs before ER exit.

In addition to TARPs and cornichon-like proteins, there is a third family of iGluR auxiliary proteins: CUB-domain containing proteins. This family includes the recently identified Neto1 (Neuropilin and tolloid like-1) and Neto2, auxiliary subunits for mammalian NMDA and kainate iGluRs (Ng et al. 2009; Zhang et al. 2009). CUB-domains are found in and named after complement subcomponent C1r/C1s, sea urchin protein Uegf, and bone morphogenetic protein BMP-1. They are conserved fragments composed of approximately 110 residues, topologically forming a "jellyroll" by 10 β-strands (Bork et al. 1993; Romero et al. 1997). CUB-domains mediate protein-protein interactions and exist in the extracellular parts of a broad spectrum of proteins, including receptors and co-receptors such as cubulin and neuropilin, metalloproteases such as BMP1 and tolloid, and adhesion molecules such as spermadhesin (Bork et al. 1993; Varela et al. 1997; Gregory et al. 2003).

Neto1 and Neto2 cDNA were isolated from mammalian retina and brain (Stohr et al. 2002). These genes encode putative single pass transmembrane proteins with two extracellular CUB-domains and one LDLa domain (Low-Density Lipoprotein class A domain). Michishita et al also isolated these genes using a signal sequence trap method, and named them as BTCL1 (Brain-specific Transmembrane protein containing CUB and LDLa domains 1) and BTCL2 (Michishita et al. 2003; Michishita et al. 2004). Sequence analyses indicated that the CUB-domains of BTCLs share significant homology with those of neuropilin-1 and neuropilin-2. Also, their expression was found to be restricted to brain region, suggesting that they are involved in neural activities. Their exact function, however, remained elusive until they were recently found to interact with NMDARs and kainate receptors and be required for receptor function (Ng et al. 2009; Zhang et al. 2009). Ng et al showed that Neto1 interacts with NR2A subunit of NMDAR and regulates the abundance of NR2A-containing NMDARs. At the behavioral level loss of Neto1 causes depressed long-term potentiation (LTP) and defective spatial learning and memory, indicating that Neto1 is a novel auxiliary subunit of the NMDAR signaling complex (Ng et al. 2009). Zhang et al established Neto2 as an accessory subunit for kainate receptors. They identified Neto2 as a kainate receptor interacting protein in a proteomic screen and found that the Neto2 surface expression is subject to regulation by kainate receptors. Additionally, kainate receptor channel properties, including decay kinetics, open probability, miniature excitatory postsynaptic currents (mEPSCs), and agonist efficacy, but interestingly, not surface expression of kainate receptors, are regulated by Neto2 (Zhang et al. 2009).

Interestingly, a *C. elegans* CUB-domain protein SOL-1 has been identified as an auxiliary subunit for GLR-1 glutamate receptors (Zheng et al. 2004), and another worm CUB-domain protein LEV-10 has been found to regulate the clustering of a subclass of acetylcholine receptors (Gally et al. 2004; Gendrel et al. 2009), strongly supporting an evolutionarily conserved role for CUB-domain-containing proteins to function as modulatory subunits of ionotropic receptors. In support of this, we recently identified another CUB-domain containing protein SOL-2, a Neto homologue, in *C. elegans* as an accessory subunit for AMPARs (Chapter 3).

In recent years, extensive efforts have been made to identify novel auxiliary subunits for AMPARs that play essential roles in fast synaptic transmission. In one study, SynDIG1 (Synapse Differentiation Induced Gene 1), a type II transmembrane protein that interact with AMPARs, was isolated from a DNA microarray based screen (Kalashnikova et al. 2010). In this study it was reported that SynDIG1 associates with AMPARs and regulates receptor levels in an activity-dependent manner (Kalashnikova et al. 2010). From a proteomic screen, another group identified a type I transmembrane protein – CKAMP44 (Cystine-Knot AMPAR Modulating Protein 44) as an AMPAR-interacting protein (von Engelhardt et al. 2010). CKAMP44 reduces AMPAR-mediated steady state currents, and interestingly, in contrast to other known auxiliary subunits, it alters the kinetics of AMPARs by enhancing desensitization and slowing recovery from desensitization. CKAMP44 also decreases the paired-pulse ratio of AMPA currents and thus negatively modulates short-term-plasticity at specific excitatory synapses (von

Engelhardt et al. 2010).

C. elegans **Neural Circuits, iGluRs and Associated Behavior**

The human brain is a huge neural network composed of about 100 billion neurons and at least a quadrillion synapses. The complexity of our brain gives us the ability to process an astronomic amount of information and carry on complex activities and behavior such as learning, memory and other cognitive activities. However, the complexity also brings daunting difficulties to the study of nervous system function. In this case, it is necessary to use simpler model organisms to better the understanding of molecular, cellular and circuit mechanisms underlying behavior. Invertebrate model animals, such as *Aplysia* (Dale et al. 1993; Frost et al. 1995; Kandel 2001), leech (Lockery et al. 1993; Lockery et al. 1993), lamprey (Grillner et al. 1995; Grillner et al. 1998) and lobster (Selverston et al. 1998), have allowed for the identification of cellular components and neural circuits that contribute to specific forms of simple behaviors, and learning and memory. In spite of the success in identifying the cellular mechanisms and neural circuitries, the molecular components that define behavior remain largely unexplored. In this case, the behaviorally and genetically tractable classical model systems, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, show their great advantages in identifying evolutionarily conserved genes that are involved in behavior.

C. elegans has been used as model animals for more than 30 years (Brenner 1974; Sulston et al. 1974). This soil nematode has almost all the rudiments of the physiological

systems, including the nervous system that is found in higher animals. But more than that, it offers many advantages for a better understanding of nervous system function. First, its nervous system is very simple. It comprises only 302 neurons but still enables relatively complicated behavior (Bargmann et al. 1998). In addition, the synaptic connections between these neurons have been completely mapped based on electron microscopic serial sections (White et al. 1986). Second, the cell lineage of *C. elegans* has been completely defined based upon the fact that cell number and cell position are invariant between individual worms. This has enabled the precise tracking of the origin and migration of neurons (Sulston et al. 1988). Third, *C. elegans* was the first completely-sequenced eukaryote genome. Moreover, it was annotated with numerous genetic perturbations useful for classical genetics. Finally, the simple anatomy and transparent body of worms provide easy *in vivo* imaging and easy access to physical manipulations. In particular, individual neurons are physiologically identifiable and easily accessible, making it possible to dissect the cuticle open and patch a specific neuron to study its electrophysiological properties (Goodman et al. 1998).

The cellular components of a number of worm neural circuits that control specific behavior have been deciphered. Worms exhibit stereotypical locomotory pattern under laboratory conditions. They mainly move forward but occasionally reverse their direction of movement briefly. This characteristic coordinated locomotory pattern with alternating forward and backward movements is important for a worm to search for food. In response to changes in environmental cues worms, however, alter their locomotory pattern. Both

coordinated and avoidance locomotory patterns are controlled by the locomotory control circuit, first defined by Chalfie and coworkers through laser ablation of specific neurons (Chalfie et al. 1985). The locomotory control circuit is composed of three groups/levels of neurons – the sensory neurons ALM, AVM, ASH and PLM that sense extrinsic signals such as mechanical, thermal and chemical stimuli, the command interneurons AVA, AVB, AVD, AVE and PVC that integrate, process and relay signals, and the motor neurons that control muscles to drive or alter movements. The locomotion control circuit can be divided into two halves – the forward part where interneurons AVB and PVC initiate forward movement, and the backward part that interneurons AVA, AVD and AVE drive backward movement (Figure 1.5).

A worm adjusts its locomotion when receiving altered sensory inputs, such as changes in tactile, chemical and thermal stimuli. In particular, when aversive stimulation is detected, avoidance responses, referring to abrupt changes in direction of movement are initiated (Bargmann et al. 1990; Kaplan et al. 1993; Wicks et al. 1995). Avoidance behavior in response to light nose touch, noxious chemicals and high osmolarity requires the polymodal sensory neuron ASH. Killing of ASH neuron leads to the elimination of both chemosensory and mechanosensory avoidance responses (Kaplan et al. 1993).

Genetic analyses have revealed that glutamate signaling is required for ASH mediated avoidance responses. The loss of *eat-4* gene, which encodes a vesicular glutamate transporter, causes the same phenotype seen in ASH-ablated worms (Lee et al. 1999). This suggests that ASH uses glutamate as neurotransmitter to pass the sensory

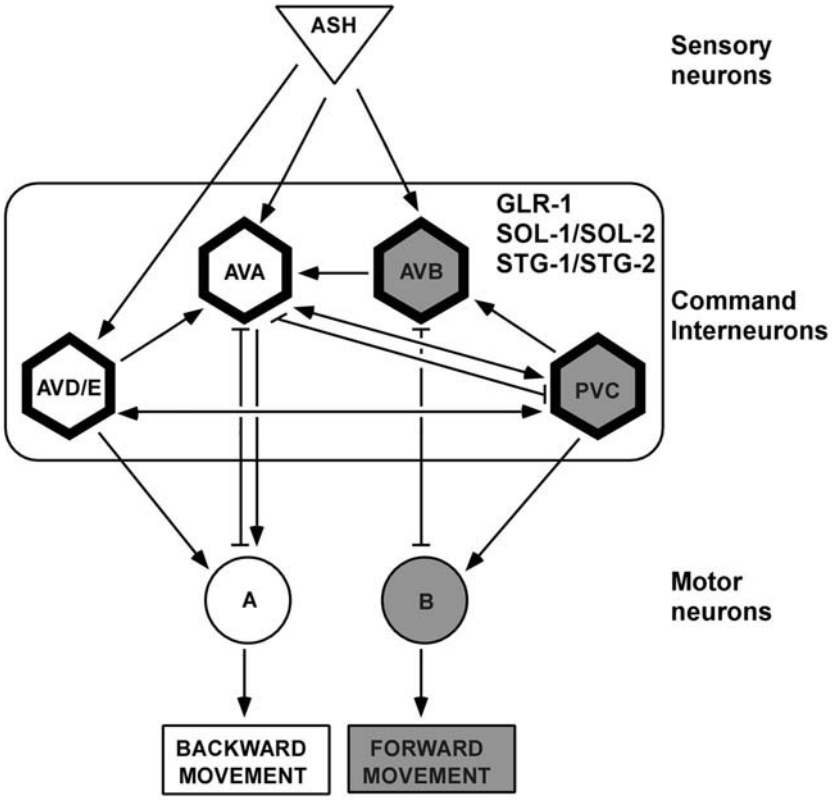

Figure 1.5 The neuronal circuit that controls locomotion in *C. elegans* (Modified from (Zheng et al. 1999)). Sensory neurons including ASH send signals to command interneurons after receiving sensory input. Command interneurons AVA, AVD/E, AVB, and PVC process the signals and send information to motor neurons to initiate forward or backward movements. Arrow heads and bars represent chemical synapses and gap junctions, respectively. GLR-1AMPA receptor and its accessory subunits SOL-1, STG-1 and STG-2 (Chapter 2), and SOL-2 (Chapter 3) are expressed in command interneurons.

signals to command interneurons. In line with this hypothesis, mutations in *glr-1* gene, which encodes a non-NMDA glutamate receptor subunit, also lead to the loss of nose touch avoidance behavior (Hart et al. 1995; Maricq et al. 1995).

The phenotypical changes in *C. elegans* locomotory pattern, such as, the changes in average forward movement duration (reversal frequency), nose touch and osmotic avoidance behavior, are extensively used in the studies presented in this dissertation to isolate the gene products that are required for GLR-1 AMPAR function and to evaluate the defective or restored behavior in mutant or rescued worms (Chapter 2 and 3).

Significance of the Research

The aim of this study was to understand what synaptic components are required for the function of glutamate receptors and how receptor function is regulated. To address this, I took advantage of the well-characterized specific glutamatergic behavior of the nematode *C. elegans*, and used powerful genetic tools to identify the accessory proteins that are indispensible for GLR-1 AMPAR function and associated behavior.

In vertebrate, there are at least 6 TARP isoforms that appear to function redundantly as AMPAR auxiliary subunits, which greatly complicates and limits the study of TARP's regulatory roles in AMPAR function, synaptic transmission and behavior. These difficulties were overcome in my study by a combined use of both reverse and forward genetics in *C. elegans*. I mutated and isolated STG-1 and STG-2, two worm TARPs. I showed that the disruption of both STG-1 and STG-2 eliminates AMPAR

mediated currents and related behavior. This is the first report of a complete knock out of the essential set of TARPs in any genome. Furthermore, this study also showed that both STG-1 and STG-2 can functionally replace vertebrate TARPs to modify receptor function, indicating an evolutionarily conserved role for TARPs. Finally, I pointed out one important difference between vertebrate TARPs and STG-1 and STG-2. Unlike vertebrate TARPs, STG-1 and STG-2 do not appear to be essential for GLR-1 AMPAR trafficking and surface expression.

From another genetic screen, I identified SOL-2, a type I transmembrane protein containing two extracelluar CUB-domains and one LDLa domain, different than previously identified SOL-1 protein that contains four extracellular CUB-domains and regulates GLR-1 gating and desensitization (Zheng et al. 2004; Walker et al. 2006; Walker et al. 2006; Zheng et al. 2006). In this study I showed that SOL-2 contributes to GLR-1 currents and behavior and co-localizes and physically associates with SOL-1 and GLR-1. More importantly, I demonstrated that SOL-2 is essential for the synaptic localization and neuronal function of the extracellular domain of SOL-1. Taken together, these results indicate that SOL-2 is required for organizing and stabilizing glutamate receptor signaling complexes and therefore qualified as an accessory subunit for GLR-1 AMPARs. Thus, this study unveiled an important organizer and scaffold of glutamate receptor signaling complexes. It also implies that the number of functional iGluRs is regulated by the interactions between CUB-domain proteins, thereby providing a novel mechanism for the regulation of iGluR function.

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CHAPTER 2

EVOLUTIONARY CONSERVED ROLE FOR TARPS IN THE GATING OF GLUTAMATE RECEPTORS AND TUNING OF SYNAPTIC FUNCTION

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Evolutionary Conserved Role for TARPs in the Gating of Glutamate Receptors and Tuning of Synaptic Function

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SUMMARY

Neurotransmission in the brain is critically dependent on excitatory synaptic signaling mediated by AMPA-class ionotropic glutamate receptors (AMPARs). AMPARs are known to be associated with Transmembrane AMPA receptor Regulatory Proteins (TARPs). In vertebrates, at least four TARPs appear to have redundant roles as obligate chaperones for AMPARs, thus greatly complicating analysis of TARP participation in synaptic function. We have overcome this limitation by identifying and mutating the essential set of TARPs in *C. elegans* (STG-1 and STG-2). In TARP mutants, AMPAR-mediated currents and worm behaviors are selectively disrupted despite apparently normal surface expression and clustering of the receptors. Reconstitution experiments indicate that both STG-1 and STG-2 can functionally substitute for vertebrate TARPs to modify receptor function. Thus, we show that TARPs are obligate auxiliary subunits for AMPARs with a primary, evolutionarily conserved functional role in the modification of current kinetics.

INTRODUCTION

Members of the TARP family are tetraspanning transmembrane proteins associated with AMPA-type glutamate receptors (AMPARs). In mice, the surface delivery of AMPARs in cerebellar granule cells is dependent on γ-2, also known as stargazin (Chen et al., 2000), the founding member of the TARP family. Thus, in stargazer mutants, no AMPA-mediated current can be evoked in cerebellar granule cells (Chen et al., 2000; Hashimoto et al., 1999). Glutamate-gated currents elsewhere in the mouse brain do not appear to be disrupted by the stargazer mutation, presumably because of the overlapping neuronal expression of multiple redundantly acting TARPs. Initially, four proteins (γ-2, γ-3, γ-4, and γ-8) were classified as TARPs based on sequence similarity and functional characteristics (Tomita et al., 2003). Recently, however, γ-7, a protein previously shown to inhibit the expression of calcium channels, was also found to have TARP-like function (Kato et al., 2007), indicating that sequence identity alone is not sufficient to predict whether a tetraspanning protein functions as a TARP.

In vitro experiments have revealed multiple roles for stargazin in vertebrates. First, stargazin serves as an obligate chaperone for AMPARs. In the absence of stargazin, AMPARs are retained in the endoplasmic reticulum and are not expressed on the cell surface (Chen et al., 2000). Second, stargazin is required for AMPAR localization to the synapse. AMPARs that are coexpressed with a stargazin that lacks a consensus PDZ domain binding motif are expressed on the cell surface but are not colocalized with the synaptic scaffolding protein PSD-95 (Schnell et al., 2002). More recent experiments have shown that stargazin changes ligand efficacy (Tomita et al., 2006) and rates of receptor deactivation and desensitization (Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005; Yamazaki et al., 2004). Much less is known about the in vivo contribution of TARPs to receptor function and synaptic signaling. Mice with a deletion mutation in the gene that encodes the γ-8 TARP have a relative decrease in extrasynaptic compared to synaptic AMPARs and a decrease in total AMPAR protein (Rouach et al., 2005).

Previous in vitro reconstitution experiments suggested that AMPARs function nearly independently of TARPs; however, more recent studies indicate that AMPARs are directly associated with a TARP (Bats et al., 2007; Nakagawa et al., 2005; Tomita et al., 2004; Walker et al., 2006a). Interestingly, one model suggests that glutamate binding causes the dissociation of the receptor from stargazin (Tomita et al., 2004). The receptor is then thought to be endocytosed after its diffusion to perisynaptic sites (Bats et al., 2007). Thus, two populations of functional AMPARs, one bound to TARPs, are hypothesized to coexist at the synapse. On the other hand, two studies suggest that all surface AMPARs are bound to TARPs (Bats et al., 2007; Nakagawa et al., 2005). In contrast to the wealth of in vitro data, the in vivo contributions of TARPs to AMPAR function have been difficult to assess due to their role as either obligate chaperones or their effects on AMPAR number and distribution. Furthermore, in vivo analysis of TARP function in mice is greatly complicated by the large number of TARPs and their widespread distribution and apparent redundant function (Kato et al., 2007; Tomita et al., 2003).

Initial efforts to identify TARP homologs in invertebrates were unsuccessful leading to the speculation that TARPs might have roles specialized for the complexities of AMPAR trafficking and

plasticity at vertebrate synapses (Vandenberghe et al., 2005). However, we recently identified stargazin-like proteins from *C. elegans*, *Drosophila*, and *Apis mellifera* (Walker et al., 2006a). Although distantly related, *C. elegans* STG-1 and vertebrate stargazin have conserved function: in reconstitution experiments, STG-1 and stargazin can act reciprocally to enhance vertebrate and *C. elegans* AMPAR-mediated currents. Invertebrate TARPs appear specifically required for AMPAR function. For example, while no glutamate-gated current can be recorded from *Xenopus* oocytes that express either the *Drosophila* GluRIA or *C. elegans* GLR-1 AMPAR subunits, large currents can be recorded when the receptors are coexpressed with invertebrate TARP proteins (Walker et al., 2006a). These TARP-dependent glutamate-gated currents are not associated with increases in receptor surface expression, suggesting that the primary role of invertebrate TARPs is to promote receptor function.

In *C. elegans*, the AMPAR subunits GLR-1 and GLR-2 (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002), together with the AMPAR auxiliary subunit SOL-1 (Zheng et al., 2004), mediate avoidance responses to tactile and osmotic stimuli. Furthermore, mutating *glr-1*, *glr-2*, or *sol-1* disrupts glutamate-gated currents in vivo (Mellem et al., 2002; Zheng et al., 2004). Reconstitution of glutamate-gated current in oocytes is dependent on coexpression of three gene products: GLR-1, SOL-1, and STG-1 (Walker et al., 2006a), suggesting that SOL-1 and STG-1, which are coexpressed with GLR-1 in the *C. elegans* nervous system, function as AMPAR auxiliary proteins (Walker et al., 2006a, 2006b).

Here, we use a genetic approach to test the requirement for STG-1 in GLR-1-mediated avoidance behaviors and currents. To our surprise, generating a deletion mutation in *stg-1* did not cause appreciable behavioral or electrophysiological phenotypes. However, by conducting a forward genetic screen for mutations that act synthetically with mutations in the *stg-1* gene, we identified a second gene (*stg-2*) that encodes a predicted TARP-like protein. GLR-1-mediated avoidance behaviors are completely disrupted in *stg-1; stg-2* double mutants, and GLR-1-mediated currents are absent, despite apparently normal surface expression of GLR-1. We also tested the function of vertebrate AMPARs expressed in transgenic worms and found that vertebrate GluR1 is expressed on the cell surface in the absence of TARP-like proteins. However, glutamate-gated currents could not be recorded unless GluR1 was coexpressed with either *C. elegans* STG-1 or vertebrate stargazin. This dependence on a TARP protein could be overcome by introducing a mutation in GluR1 that prevented receptor desensitization. Thus, our genetic and electrophysiological analyses point to an essential, evolutionarily conserved role for TARPs in regulating AMPAR function.

RESULTS

To test STG-1's role in nervous system function, we used standard techniques to first generate a transposon insertion in the *stg-1* gene and then to detect a rare imprecise excision of the transposon that deleted almost the entire *stg-1* coding sequence (Figures 1A and 1B). *stg-1(ak104)* mutants were viable and had no obvious morphological or movement abnormalities. To assess the involvement of STG-1 in AMPAR-mediated neurotransmission, we tested the performance of *stg-1* mutants in behaviors mediated by the GLR-1 AMPAR. *C. elegans* recoils in response to mechanostimulation of the anterior tip of the head (nose-touch response), and this avoidance behavior is dependent on glutamatergic neurotransmission that activates GLR-1 (Hart et al., 1995; Lee et al., 1999; Maricq et al., 1995). However, we did not find that nose-touch avoidance was disrupted in *stg-1* mutants (Figure 1C). We also examined the hyperreversal or "lurching" phenotype of transgenic worms that expressed a GLR-1 gain-of-function variant, GLR-1(A687T) (Figure 1D). The average duration of forward movement before reversing is greatly reduced in lurcher worms compared to wild-type (Zheng et al., 1999), and the suppression by the *stg-1(ak104)* mutation was only a small fraction of that previously observed for the *sol-1* mutation (Zheng et al., 2004). To directly address whether AMPAR-mediated currents were disrupted in *stg-1* mutants, we used conventional whole-cell patch-clamp techniques (Francis and Maricq, 2006) to record glutamate-gated currents from AVA, an interneuron that is required for backward movement. In contrast to *glr-1* and *sol-1* mutants (Zheng et al., 2004), we found no appreciable differences in glutamate-gated currents between wild-type and *stg-1* mutants (Figures 1E and 1F).

stg-1; stg-2 Double Mutants Have Synergistic Defects

Considering the importance of STG-1 in reconstitution experiments and its coexpression with GLR-1 in the nervous system (Walker et al., 2006a), we considered the possibility that additional TARP proteins may be coexpressed with GLR-1. We identified several additional genes that encode proteins with weak sequence identity to STG-1, including *clc-3*, *clc-4*, and *f53b3.5* (Figure 2A). However, the *stg-1; clc-3* double mutant had no obvious defects in GLR-1-dependent behaviors, and *clc-4* is not expressed in the nervous system (data not shown). Also, we were unable to record glutamate-gated currents from *Xenopus* oocytes that coexpressed either CLC-3 or F53B3.5 with SOL-1 and GLR-1 (data not shown). Therefore, we used a forward genetic approach to identify additional gene products that may contribute to glutamatergic neurotransmission. We reasoned that screening for genes that acted in concert with *stg-1* might circumvent the potential problem of redundancy. Thus, we screened for synthetic mutations—those that individually did not suppress the GLR-1(A687T) lurcher phenotype but did cause suppression in worms that also contained the *stg-1* mutation.

Our screen identified two mutations that were synthetic with *stg-1* and failed to complement each other. We mapped the mutations to a small interval on LG X and, using standard cosmid rescue techniques, identified mutations in an open reading frame in cosmid F12D9 (Figure 2B). F12D9.1b is predicted to encode a 279 amino acid (aa) protein with no identity to known proteins (wormbase.org). Our analysis of the genome and of the corresponding cDNA indicates that the coding sequence, which we have named *stg-2*, actually extends an additional 234 bp, encoding a 357 aa protein. Like other TARPs and γ-subunits, STG-2 has 4 predicted transmembrane domains but has rather low sequence identity with either *C. elegans* STG-1 or vertebrate stargazin (Figures 2A and 2C). The *stg-2(ak134)* allele contains a nonsense mutation following aa 108, suggesting that this

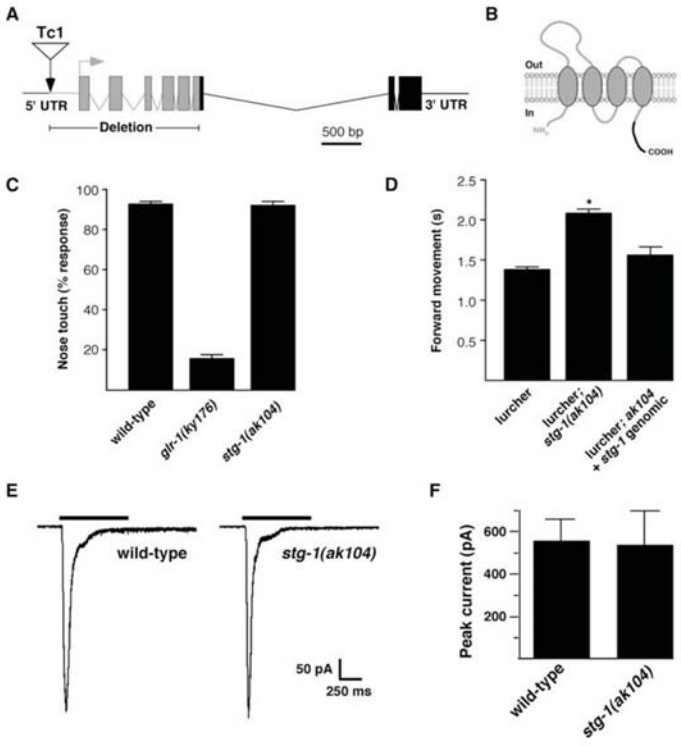

Figure 1. The STG-1 and STG-2 TARP-like Proteins Differentially Affect GLR-1-Mediated Behaviors

(A) Genomic organization of the *stg-1* locus with exons and introns represented as boxes and lines, respectively. The black arrow indicates the site of the Tc1 insertion, and gray shows the region deleted by its imprecise excision.

(B) Membrane topology of the predicted STG-1 protein showing the four transmembrane domains and the intracellular N- and C-terminal domains. Gray represents the region deleted in the *stg-1(ak104)* allele.

(C) The nose touch response in wild-type, n = 16; *glr-1(ky176)* mutants, n = 12; and *stg-1(ak104)* mutants, n = 15.

(D) Average duration of forward movement in transgenic lurcher worms, n = 10; lurcher; *stg-1(ak104)* mutants, n = 10; and lurcher; *stg-1(ak104)* mutants that expressed an extrachromosomal wild-type copy of *stg-1*, n = 4. *significantly different from lurcher worms, $p < 0.001$.

(E) Glutamate-gated currents in response to 3 mM glutamate application recorded from the AVA interneuron in wild-type worms (left) and *stg-1(ak104)* mutants (right).

(F) Mean peak glutamate-gated current amplitude in AVA of wild-type, n = 7, and *stg-1(ak104)* mutants, n = 3.

Error bars indicate SEM.

mutation is a null. The *stg-2(ak138)* mutation contains both a missense mutation and a small deletion (removing aa 26–34), resulting in a premature stop codon; however, a plausible initiating methionine exists at aa 45 (Figure 2C). Neither mutation alone significantly suppressed the hyperreversal phenotype of lurcher worms. In contrast, the duration of forward movement was increased significantly in *stg-1* mutants that also carried either allele of *stg-2* (Figure 2D). This effect was more pronounced for *stg-1; stg-2(ak134)* double mutants, consistent with the notion that *ak134* represents a null allele. For this reason, we focused most of our subsequent analyses using this allele. The *stg-1; stg-2* double mutants incompletely suppressed lurching behavior, consistent with previous evidence that GLR-1(A687T) maintains some function in the absence of STGs (Walker et al., 2006a). Lurching behavior was restored in transgenic lurcher; *stg-1(ak104); stg-2(ak134)* double mutants that overexpressed either STG-1 or STG-2 (Figure 2D).

To address the contribution of *stg-2* to behavior, we outcrossed the mutant worms to remove the GLR-1(A687T) transgene. Compared to *glr-1(ky176)* null mutants, which are slow to avoid osmotic stimuli, *stg-1* or *stg-2* single mutants responded with delays comparable to wild-type worms (Figure 2E). In contrast, *stg-1(ak104); stg-2(ak134)* double mutants were indistinguishable from *glr-1* mutants, and normal osmotic avoidance behavior was restored in transgenic *stg-1; stg-2* mutants that expressed wild-type copies of either *stg-1* (*stg-1* genomic) or *stg-2* (*stg-2* genomic) (Figure 2E). *stg-1; stg-2* double mutants also showed comparable defects to *glr-1* mutants in nose touch avoidance (Figure 2F). However, in this assay, we found that *stg-2* mutants were also significantly different from wild-type worms, whereas *stg-1* mutants were indistinguishable from wild-type (Figure 2F). Normal behavior was restored in transgenic *stg-2(ak134)* mutants that expressed wild-type copies of the *stg-2* gene.

STG-1 and STG-2 Have Overlapping but Distinct Expression Patterns

The synthetic phenotypes that we observed in our behavioral analysis suggested that most neurons that express GLR-1 should also express both STG-1 and STG-2; however, the disrupted nose-touch response of *stg-2*, but not *stg-1*, mutants implied that a subset of neurons required for nose-touch avoidance may differentially express the STG proteins. To test this hypothesis, we generated transgenic worms that coexpressed GFP under the regulation of the *nmr-1* promoter (*Pnmr-1*::GFP) and the mCherry protein driven by either the *stg-1* (*Pstg-1*:mCherry) or *stg-2* (*Pstg-2*::mCherry) promoter. The *nmr-1* promoter drives GFP expression in a subset of GLR-1-expressing interneurons,

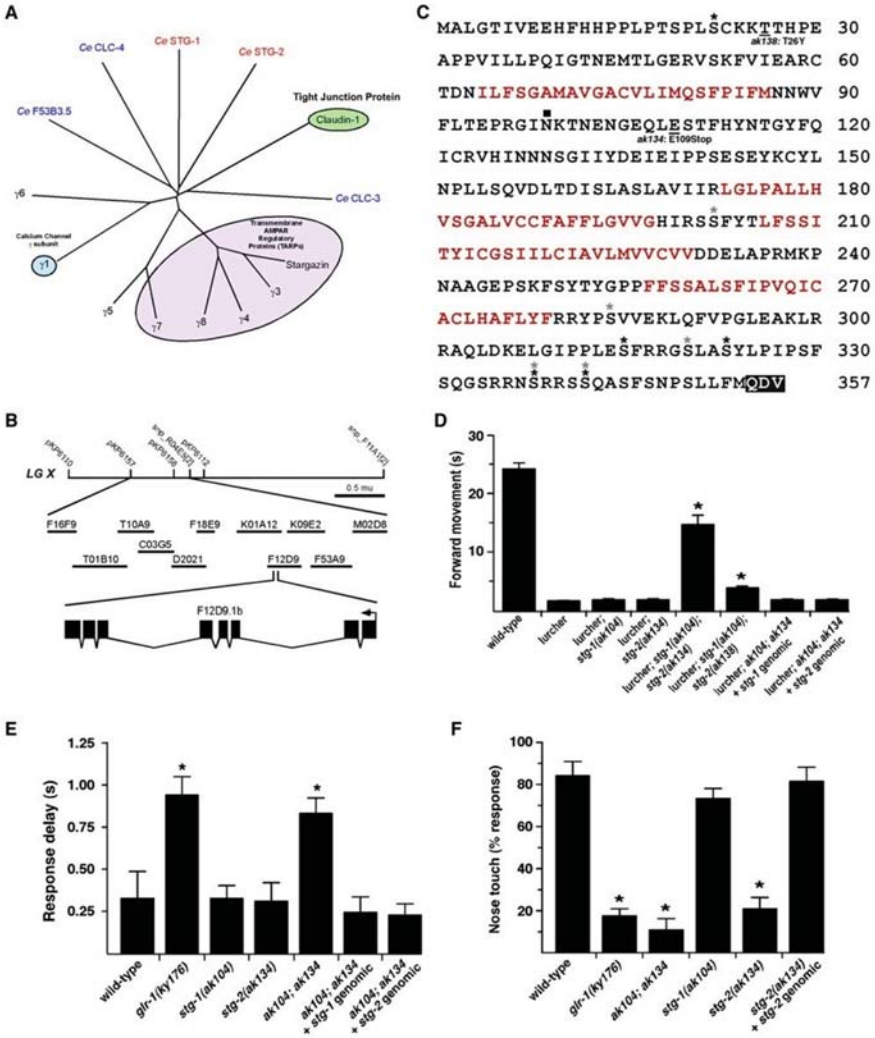

Figure 2. *stg-2* Encodes a TARP Protein Required for GLR-1-Mediated Behavior

(A) Phylogenetic tree of vertebrate TARPs; the calcium channel subunit, γ-1; the tight junction protein, claudin-1; and *C. elegans* STG-1, STG-2, CLC-3, CLC-4, and F53B3.5 (adapted from Tomita et al., 2003).

(B) Region of linkage group (LG) X showing the single nucleotide polymorphisms used to map the *ak134* and *ak138* mutations (top). Cosmids that cover the region are shown. The open reading frame F12D9.1b constitutes the *stg-2* locus, the genomic organization of which is shown with boxes and lines representing exons and introns, respectively (bottom).

(C) Predicted protein sequence encoded by the *stg-2* gene. Indicated are putative transmembrane domains (red text), N-linked glycosylation site (filled square), PKA phosphorylation sites (gray asterisks), and PKC phosphorylation sites (black asterisks). The sites of the *ak134* and *ak138* mutations are underlined, and the black box highlights a putative noncanonical PDZ-domain binding motif.

(D) Average duration of forward movement for wild-type (n = 15); transgenic lurcher worms (n = 15); and the following mutants that expressed the lurcher transgene: *stg-1(ak104)* single mutant (n = 15), *stg-2(ak134)* single mutant (n = 8); *stg-1(ak104); stg-2(ak134)* double mutant (n = 7); *stg-1(ak104); stg-2(ak138)* double mutant (n = 7). STG-1 (n = 7) and STG-2 (n = 7) genomic rescue are also shown. *significantly different from lurcher, $p < 0.001$.

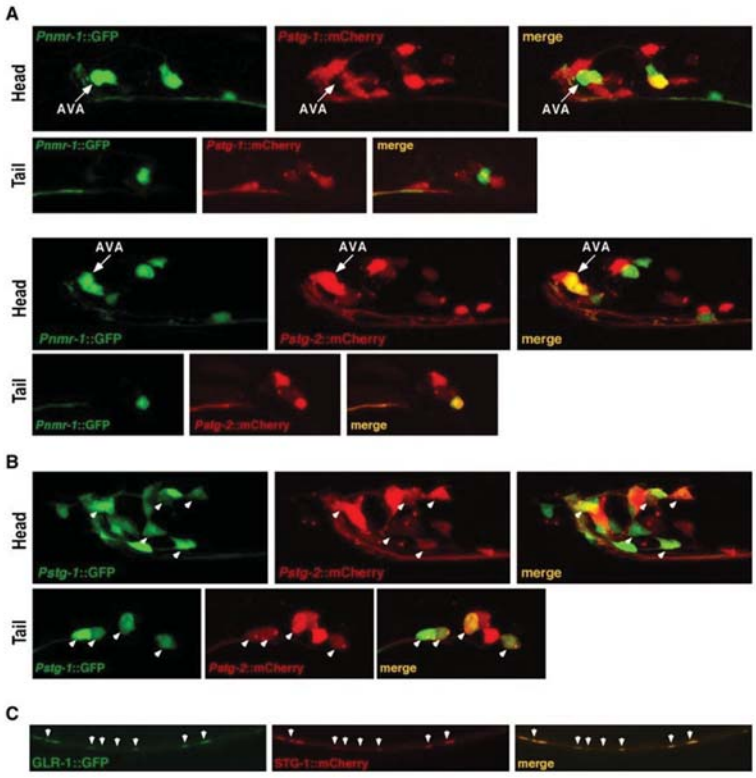

Figure 3. STG-1 and STG-2 Are Differentially Expressed in the Nervous System

(A) Confocal images of transgenic worms that expressed *Pnmr-1*::GFP and *Pstg-1*::mCherry or *Pnmr-1*::GFP and *Pstg-2*::mCherry. Head and tail regions only. Arrows point to the AVA interneuron.

(B) Confocal images of a transgenic worm that expressed *Pstg-1*::GFP and *Pstg-2*::mCherry. Arrowheads indicate a subset of the cells that coexpress GFP and mCherry.

(C) Confocal images of the ventral nerve cord in a transgenic worm that expressed functional GLR-1::GFP and STG-1::mCherry both under the regulation of the *glr-1* promoter. Arrowheads indicate puncta thought to represent postsynaptic sites.

including most of the command interneurons that regulate avoidance responses (Brockie et al., 2001a, 2001b). Using confocal microscopy to examine GFP and mCherry expression in transgenic worms, we found that most of the interneurons that expressed *Pnmr-1*::GFP also appeared to express both *Pstg-1*::mCherry and *Pstg-2*::mCherry, with the notable exception of the AVA command interneuron (Figure 3A). STG-2, but not STG-1, was strongly expressed in AVA, which is required for backward avoidance responses (Chalfie et al., 1985; de Bono and Maricq, 2005). Both *Pstg-1*::mCherry and *Pstg-2*::mCherry expression were more widespread than *Pnmr-1*::GFP and were exclusively expressed in neurons. Coexpressing *Pstg-1*::GFP with *Pstg-2*::mCherry revealed considerable overlap in their expression patterns (Figure 3B). We also generated transgenic worms that coexpressed functional full-length STG-1::mCherry and GLR-1::GFP both under the regulation of the *glr-1* promoter. The fusion proteins colocalized at punctate structures in the ventral cord that are thought to represent postsynaptic sites (Figure 3C). This suggests that STG-1 and GLR-1 form part of a signaling complex at synapses. We were unable to confidently

(E) The delay in the response to hyperosmotic stimuli in wild-type, n = 9; *glr-1(ky176)*, n = 8; *stg-1(ak104)*, n = 8; *stg-2(ak134)*, n = 8; *stg-1(ak104); stg-2(ak134)*, n = 18; *ak104; ak134 + stg-1* genomic, n = 7; and *ak104; ak134 + stg-2* genomic, n = 10. *significantly different from wild-type, $p < 0.01$.

(F) The nose-touch response in wild-type, n = 3; *glr-1(ky176)*, n = 3; *stg-1(ak104); stg-2(ak134)*, n = 3; *stg-1(ak104)*, n = 4; *stg-2(ak134)*, n = 3; and *stg-2(ak134) + stg-2* genomic, n = 6. *significantly different from wild-type, $p < 0.005$.

Error bars indicate SEM.

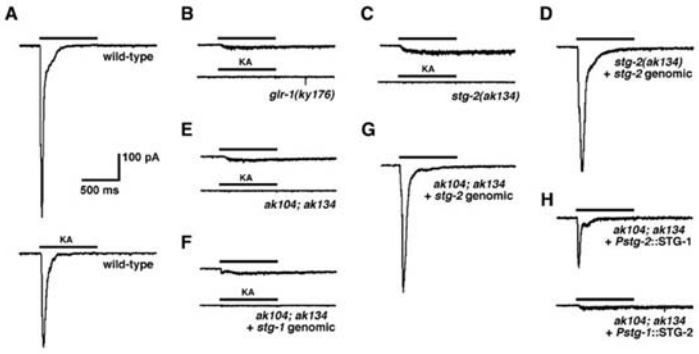

Figure 4. *stg-2* Is Required for GLR-1-Mediated Glutamate-Gated Currents

(A–H) Current responses to 1 or 3 mM glutamate or 100 μM kainate (KA) measured in the AVA interneuron in wild-type, mutant, and transgenic mutant worms.

assess STG-2 localization because the reporter fusions for the STG-2 protein were not functional.

GLR-1-Mediated Currents Are Not Detected in *stg-1; stg-2* Double Mutants

To directly examine GLR-1 function in wild-type and mutant worms, we used in vivo patch-clamp recording techniques to record glutamate-gated currents from the AVA interneuron. In wild-type worms, pressure application of glutamate evoked an inward current (537 ± 85 pA, n = 7) that desensitized in the continued presence of glutamate (Figure 4A). At least two classes of receptors contribute to the glutamate-gated current: rapidly activating AMPARs that contain the GLR-1 subunit and slower, rectifying NMDA-type receptors that contain the NMR-1 subunit (Brockie et al., 2001b; Mellem et al., 2002; Zheng et al., 2004); the NMDA receptor-mediated component is the predominant current observed in *glr-1* mutants (Figure 4B). GLR-1-mediated current can be selectively activated using the agonist kainate (342 ± 32 pA, n = 4) (Figure 4A), and no kainate-gated current is detected in *glr-1* mutants (Figure 4B). In *stg-1* mutants, glutamate-gated currents appeared indistinguishable from those recorded in wild-type worms (Figure 1E). In contrast, only the NMDA component of the glutamate-gated current was recorded in *stg-2* mutants, and no kainate-gated current was detected (Figure 4C). Apparently, normal currents were recorded in transgenic *stg-2* mutants that expressed a wild-type copy of *stg-2* (*stg-2* genomic) (459 pA ± 117, n = 4) (Figure 4D). *stg-1; stg-2* double mutants appeared indistinguishable from *stg-2* mutants and *glr-1* mutants (Figure 4E), providing additional evidence that rapid-glutamate-gated currents in AVA were primarily dependent on STG-2. Glutamate-gated current was restored in transgenic *stg-1; stg-2* double mutants that expressed a wild-type copy of *stg-2* (453 pA ± 28, n = 3), but rescue was not observed when expressing STG-1 under control of its native promoter (Figures 4F and 4G). We did note a very small, rapid inward current that may be a consequence of overexpressing the *stg-1* transgene (Figure 4F). These data indicate that GLR-1-mediated glutamate-gated currents in AVA are primarily dependent on STG-2, a finding that is consistent with the differential expression of STG-1 and STG-2 in AVA (Figure 3).

A question raised by these results is whether STG-1 can functionally substitute for STG-2 in AVA. To address this, we recorded glutamate-gated currents from transgenic *stg-1; stg-2* double mutants that expressed STG-1 or STG-2 under control of the *stg-2* and *stg-1* promoters, respectively, i.e., *Pstg-2*::STG-1 or *Pstg-1*::STG-2. These promoter swap experiments showed that rapid, GLR-1-mediated currents were detected when STG-1 was expressed under control of the *stg-2* promoter (Figure 4H). Our data show that either STG-1 or STG-2 was competent to promote glutamate-gated currents in AVA and that the STG proteins function cell-autonomously. However, the current measured in transgenic animals that express *Pstg-2*::STG-1 was smaller than that for wild-type (73 ± 27 pA, n = 5; $p < 0.01$). This difference may reflect variability in transgenic expression or indicate that additional gene products contribute to iGluR function in AVA.

GLR-1 Is Expressed on the Cell Surface in the Absence of Both STG-1 and STG-2

In cerebellar granule cells, the surface expression of AMPARs is greatly reduced in stargazin mutants (Chen et al., 2000). One explanation for the phenotype of *stg-1; stg-2* double mutants is that GLR-1 is not present on the cell surface. To address this possibility, we examined GLR-1 expression in transgenic worms that expressed a functional hemagglutin (HA) epitope-tagged GLR-1::GFP under control of the *glr-1* promoter (HA::GLR-1::GFP). Using anti-HA antibodies under nonpermeabilized conditions (see Experimental Procedures), we found similar levels of surface expression in wild-type worms, *stg-1* or *stg-2* single mutants, and *stg-1; stg-2* double mutants (Figures 5A and 5B). We also found that vertebrate stargazin did not increase surface expression of GLR-1 in *Xenopus* oocytes and that neither STG-1 nor STG-2 increased the surface expression of vertebrate GluR1 (Figures S1A and S1B available online). These data are consistent with

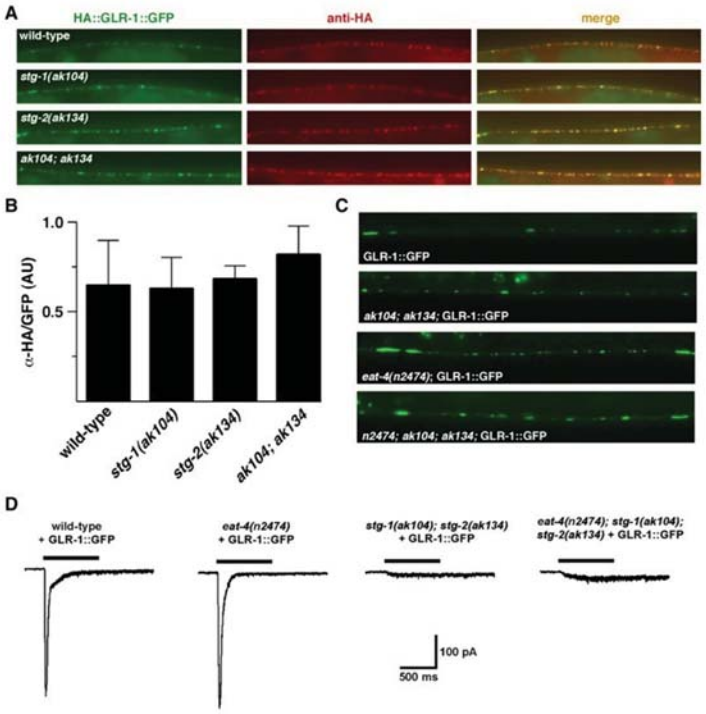

Figure 5. GLR-1 Surface Expression Is Independent of Both STG-1 and STG-2

(A) Images of transgenic wild-type and mutant worms that expressed HA::GLR-1::GFP under the regulation of the *glr-1* promoter. GFP (left) and anti-HA staining (middle) in the neural processes of the ventral cord are shown. Merged image, right.

(B) The ratio of anti-HA staining to GFP fluorescence in the ventral cord of transgenic worms shown in (A), arbitrary units (AU). Wild-type, n = 3; *stg-1(ak104)*, n = 7; *stg-2(ak134)*, n = 6; *ak104; ak134*, n = 9.

(C and D) Images of GFP expression in the ventral cord (C) and current response to 3 mM glutamate application (D) in transgenic wild-type and mutant worms that overexpressed GLR-1::GFP.

Error bars indicate SEM.

a previous report that showed GLR-1 is delivered to the cell surface when ectopically expressed in muscle cells of transgenic worms in the absence of STG-1, STG-2, and SOL-1 (Walker et al., 2006a). Together, these results suggest that the GLR-1-mediated behavioral and electrophysiological defects observed in *stg-1; stg-2* double mutants are not due to altered GLR-1 surface expression and that STG-1 and STG-2 are required for GLR-1 function.

To further address this question, we characterized GLR-1 expression and glutamate-gated currents in worms that lacked a *C. elegans* vesicular glutamate transporter, EAT-4 (Lee et al., 1999). We previously demonstrated that the chronic lack of glutamatergic neurotransmission in *eat-4* mutants caused compensatory postsynaptic changes in GLR-1 (Grunwald et al., 2004). Thus, both the abundance of GLR-1::GFP in neuronal processes and the magnitude of GLR-1-mediated currents measured in vivo were increased in *eat-4* mutants (315 pA ± 85; n = 2) compared to wild-type worms (245 pA ± 48; n = 2) (Figures 5C and 5D) (Grunwald et al., 2004). The abundance of GLR-1::GFP also appeared to be increased in *eat-4; stg-1; stg-2* triple mutants compared to *stg-1; stg-2* double mutants (Figure 5C). However, despite this, we could not detect GLR-1-mediated current in the *eat-4; stg-1; stg-2* triple mutants, even when GLR-1 was overexpressed (n = 3; Figure 5D). These results indicate that both normal and compensatory trafficking and surface expression of GLR-1 occurs independently of STG-1 and STG-2 and that increased GLR-1 expression does not overcome the functional dependence on *C. elegans* TARP proteins.

STG-1 and STG-2 Differentially Modify the Kinetics of GLR-1 AMPA Receptors

Information processing at synapses is greatly influenced by the time course of the synaptic current. To address whether STG-1 and STG-2 have different effects on the kinetics of GLR-1-mediated currents and, thus, GLR-1 function, we recorded glutamate-gated currents in response to rapid application of glutamate. For

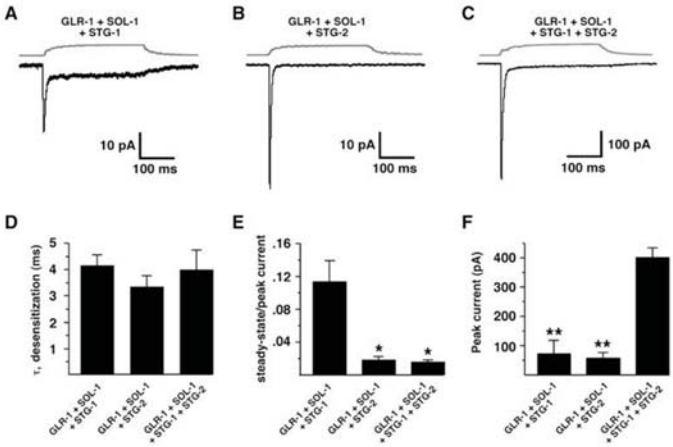

Figure 6. TARPs Differentially Regulate *C. elegans* GLR-1 and Vertebrate GluR1 Receptor Desensitization

(A–C) Currents measured in response to the rapid application of 3 mM glutamate in cultured muscle cells dissociated from transgenic *stg-1(ak104); stg-2(ak134)* worms that ectopically expressed various combinations of GLR-1, SOL-1, STG-1, and STG-2 in body wall muscle cells. The gray trace represents the open-tip potential.

(D–F) Rate of receptor desensitization (D), steady-state to peak current ratio (E), and mean peak current (F) for currents measured in dissociated muscle cells described in (A)–(C). GLR-1 + SOL-1 + STG-1, n = 4; GLR-1 + SOL-1 + STG-2, n = 6; GLR-1 + SOL-1 + STG-1 + STG-2, n = 4. *significantly different from GLR-1 + SOL-1 + STG-1, $p < 0.01$. **significantly different from GLR-1 + SOL-1 + STG-1 + STG-2, $p < 0.01$.

Error bars indicate SEM.

better control of the genetic background, we expressed GLR-1 and SOL-1 along with either STG-1 or STG-2 in the muscle cells of transgenic *stg-1; stg-2* double mutants. In *C. elegans*, excitatory neuromuscular transmission is mediated by acetylcholine, not glutamate, and no endogenous glutamate-gated currents are present in muscle cells (Walker et al., 2006b). We recorded glutamate-gated currents from cultured muscle cells dissociated from transgenic worms that ectopically expressed components of the GLR-1/SOL-1/STG receptor complex in body wall muscle cells. We previously demonstrated that reconstitution of glutamate-gated current in muscle depends on STG-1 (Walker et al., 2006a). We now find that the time course of glutamate-gated current is dramatically influenced by the STG subtype that is coexpressed with GLR-1 and SOL-1 (Figure 6). Thus, when STG-1 was coexpressed with GLR-1 and SOL-1, glutamate-gated currents incompletely desensitized (Figures 6A, 6D, and 6E), whereas full and rapid desensitization was observed when STG-2 was coexpressed (Figures 6B, 6D, and 6E). Interestingly, when both STG-1 and STG-2 were coexpressed, the peak glutamate-gated current was almost 10-fold greater than that observed with coexpression of either STG-1 or STG-2 alone (Figures 6C and 6F), suggesting perhaps that all four proteins may interact to form a signaling complex.

STG-2 Promotes Glutamate-Gated Currents Mediated by Invertebrate and Vertebrate iGluRs

To examine whether STG-2 can promote glutamate-gated currents in reconstitution experiments, we measured glutamate-gated currents from *Xenopus* oocytes that expressed combinations of *C. elegans* GLR-1, SOL-1, STG-1, and STG-2. As previously observed (Walker et al., 2006a; Zheng et al., 2006), no glutamate-gated current was detected in oocytes that only coexpressed GLR-1 and SOL-1. In contrast, coexpression with STG-1 or STG-2, or both STG-1 and STG-2, was sufficient to promote glutamate-gated currents (Figures 7A and 7B). In contrast to rapid perfusion experiments (Figure 6), we did not find increased current when both STG-1 and STG-2 are coexpressed. This apparent difference may be secondary to the slow kinetics of drug application when recording from oocytes. We also found that either STG-1 or STG-2 was competent to promote vertebrate GluR1-mediated currents (Figures 7C and 7D), although the *C. elegans* proteins were less efficacious than vertebrate stargazin, with STG-2 having the smallest effect. The apparent difference between STG-1 and STG-2 may reflect differences in protein expression or receptor kinetics. Glutamate-gated currents in oocytes that coexpressed GluR1 and STG-2 desensitized with a time course similar to that observed in oocytes that coexpressed GluR1 and vertebrate stargazin. In contrast, glutamate-gated currents in oocytes that coexpressed GluR1 and STG-1 only partially desensitized (Figure 7C). Oocytes that coexpressed GluR1, STG-1, and STG-2 desensitized with a time course similar to that observed in oocytes that coexpressed GluR1 and STG-1 (Figures 7E and 7F).

Vertebrate GluR1 Function Is Dependent on TARP Proteins

Glutamate-gated currents can be recorded from *Xenopus* oocytes injected with cRNA encoding vertebrate GluR1; however, the magnitude of the current is increased with the coexpression of stargazin (Chen et al., 2003). The interpretation of this result is that overexpression of GluR1 can bypass the need for stargazin. Considering that TARPs from different species (and within species) have low sequence identity but conserved function, an alternative hypothesis is that some protein activity in *Xenopus* oocytes promotes GluR1 function. To test this hypothesis, we expressed GluR1 in muscles of transgenic worms and recorded current in response to pressure application of glutamate. We did not record fast glutamate-gated currents from muscles that overexpressed functional GFP::GluR1 alone (Figure 8A), although GFP::GluR1 was expressed at the cell surface (Figure 8B). Occasionally, we recorded very small, slow currents (<10 pA). In contrast, we observed large, fast glutamate-gated currents when GFP::GluR1 was coexpressed with stargazin (484 pA ± 187, n = 6), and smaller currents when coexpressed with *C. elegans*

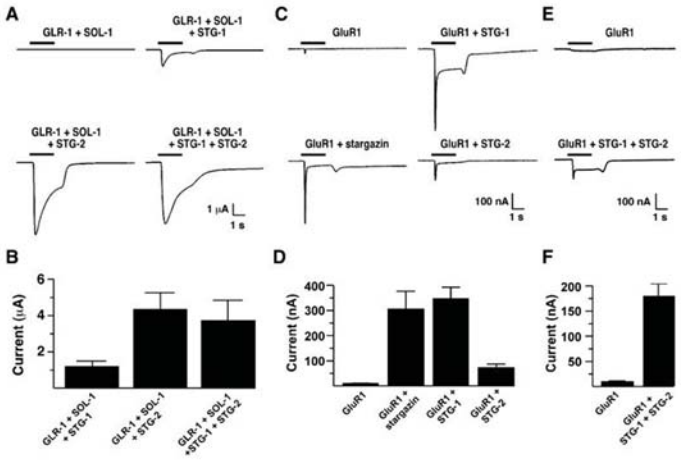

Figure 7. Both STG-1 and STG-2 Can Partially Substitute for Vertebrate Stargazin in Heterologous Cells

(A, C, and E) Currents measured in response to 1 mM glutamate application in *Xenopus* oocytes that expressed various combinations of GLR-1, SOL-1, STG-1, and STG-2 (A), or vertebrate GluR1, stargazin, STG-1, and STG-2 (C and E) cRNAs were injected at 8.3 ng (GLR-1, SOL-1, total STG), 0.1 ng (GluR1), 0.56 ng (stargazin). (B, D, and F) Mean peak current amplitude. (B) GLR-1 + SOL-1 + STG-1, n = 12; GLR-1 + SOL-1 + STG-2, n = 12; GLR-1 + SOL-1 + STG-1 + STG-2, n = 7. (D) GluR1, n = 12; GluR1 + stargazin, n = 11; GluR1 + STG-1, n = 12; GluR1 + STG-2, n = 11. (F) GluR1, n = 4; GluR1 + STG-1 + STG-2, n = 6. Error bars indicate SEM.

STG-1 (56 pA ± 17, n = 7; Figure 8A). Surface delivery of GFP::GluR1 appeared equivalent in the presence or absence of either stargazin or STG-1 (Figure 8B). Importantly, any possible differences in surface expression cannot explain the large relative differences in glutamate-gated current observed with coexpression of stargazin or STG-1. The larger current observed with stargazin may reflect a greater affinity for vertebrate GluR1. We also found that introducing a point mutation into GluR1 (L507Y) that blocks desensitization (Stern-Bach et al., 1998) restored glutamate-gated current in the absence of either stargazin or STG-1 (403 pA ± 33, n = 4; Figure 8A), providing further evidence for stargazin-independent surface expression of GluR1. Together, these results indicate that the primary evolutionarily conserved role for TARPs is to promote the function of iGluRs.

DISCUSSION

Our study provides a comprehensive analysis of AMPAR function in mutants devoid of TARP function. Using both reverse and forward genetic approaches, we have identified and deleted two TARP proteins in *C. elegans*, STG-1 and STG-2. In *C. elegans*, we could evaluate the unique contribution of each TARP to receptor function without the confounding variable of possible compensatory TARPs. Furthermore, by characterizing the in vivo kinetic properties of AMPARs in the absence of TARPs, we were able to shed light on the possible role of TARPs at synapses. Although STG-1 and STG-2 are distantly related by primary sequence to vertebrate TARPs, both can functionally substitute for vertebrate TARPs in reconstitution experiments. In *stg-1; stg-2* double mutants, GLR-1 receptors are expressed on the cell surface; however, no AMPAR-mediated current is detected, and the behavioral phenotype mimics that of *glr-1* mutants. Our experiments have identified the minimal set of TARP proteins in *C. elegans* that are required for normal glutamatergic communication and demonstrate that TARPs have essential, evolutionarily conserved roles in regulating the kinetics of AMPARs.

TARPs Are Required for AMPAR Function in *C. elegans*

In *stg-1; stg-2* double mutants, no in vivo glutamate-gated current could be elicited by pressure application of glutamate. Reconstitution experiments in transgenic *C. elegans* muscle cells provided similar results. Even when using rapid perfusion techniques, we did not observe fast glutamate-gated currents in the absence of TARPs. The lack of current in *stg-1; stg-2* double mutants could not be explained by changes in surface expression of GLR-1 as both the distribution and surface expression of GLR-1 in *stg-1; stg-2* double mutants appeared indistinguishable from that in wild-type worms. The first characterized TARP has significant roles in the surface expression and localization of AMPARs (Chen et al., 2000). Later, three other TARPs were discovered, each with an equivalent ability to promote surface expression (Tomita et al., 2003). However, even the role of vertebrate TARPs now appears more complicated, e.g., a new TARP (γ-7) is quite divergent in sequence and whether it participates in surface delivery or receptor function has not yet been established (Kato et al., 2007). In contrast, there is no apparent role for TARPs in the trafficking or surface expression of AMPARs in *C. elegans*. These differences may reflect evolutionary changes. For example, functions in trafficking and surface expression may have been gained or lost during evolution. Reconstitution experiments in *Xenopus* oocytes and cell lines show that functional AMPARs are expressed on the cell surface in the absence of stargazin, suggesting stargazing-independent trafficking. However, the genetic background of these heterologous systems has not been established. At least five vertebrate proteins (γ-2, γ-3, γ-4, γ-8, and γ-7) have functional effects on AMPARs, and these proteins are widely expressed. It is possible that heterologous cells express endogenous TARPs that promote receptor surface delivery and function. There is precedent for this notion. The NR1 NMDA receptor subunit was cloned by functional expression in *Xenopus* oocytes (Moriyoshi et al., 1991); however, NMDA receptors are now known to be heteromeric receptors with NR2 subunits, and NR1 does not bind

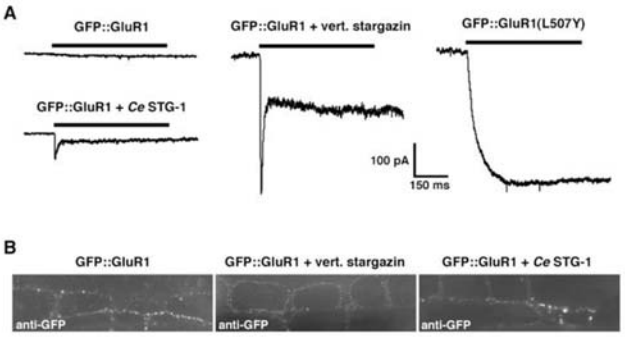

Figure 8. Vertebrate GluR1 Function Is Dependent on TARP Proteins

(A) Currents measured in response to pressure application of 3 mM glutamate to body wall muscle cells that expressed vertebrate GFP::GluR1 alone, GFP::GluR1(L507Y) alone, or GFP::GluR1 coexpressed with either *C. elegans* (*Ce*) STG-1 or vertebrate stargazin under the regulation of the muscle specific *myo-3* promoter.

(B) Images of anti-GFP staining in transgenic wild-type worms that expressed GFP::GluR1 in muscle cells.

glutamate (Dingledine et al., 1999), strongly suggesting that *Xenopus* oocytes must express endogenous NR2-like proteins.

Vertebrate GluR1 Function Depends on TARPs

We have demonstrated that, independent of any TARP effects on surface expression or localization of vertebrate AMPARs, TARPs are required for receptor function. Our reconstitution experiments in *C. elegans* show that vertebrate GluR1 is trafficked to the surface but that glutamate-gated currents cannot be measured in the absence of a TARP, whether vertebrate or invertebrate, thus demonstrating that TARPs have an evolutionarily conserved role in AMPAR function independent of other roles. A mutation that prevents GluR1 desensitization restores function in the absence of exogenous TARPs, suggesting that TARPs act to regulate the desensitization of the receptor.

TARPs Differentially Regulate the Rate of GLR-1 Desensitization

Because all AMPAR-mediated current was eliminated in *stg-1; stg-2* double mutants, we could evaluate the unique contribution of each TARP to receptor function without the confounding variable of possible compensatory TARPs. We found that the rate and extent of glutamate-gated current desensitization was greater with STG-2 than with STG-1. Desensitization is an evolutionarily conserved feature of ligand-gated receptors. We propose that the differential neuronal expression of STG-1 and STG-2 may optimize the bandwidth of synaptic transmission by tuning the kinetics of glutamate-gated currents. The diversity of TARP effects on desensitization can be appreciated by comparing the varying impact of STG-1, stargazin (Figures 7 and 8), and *Apis* STG1 (Walker et al., 2006b) on currents mediated by vertebrate GluR1. At vertebrate synapses, TARPs may similarly modify synaptic signaling and thus behavior.

The Diversity of TARPs

The conserved function of vertebrate and invertebrate TARPs despite the low amino acid identity suggests that 3-dimensional structure is conserved and retains functional effects on quite divergent AMPARs (insect, nematode and vertebrate). The functional properties of AMPARs are influenced by subunit composition, alternative splicing, RNA editing, phosphorylation state, and in dramatic fashion by association with one or more TARPs. Thus, this tremendous combinatorial complexity in receptor function may contribute to the synaptic processing of information. The percent identity between *C. elegans* STG-1 and STG-2 is almost the same as the identity between STG-1 and vertebrate stargazin, highlighting the difficulty in estimating the true number of TARPs in a genome. In vertebrates, the original TARP family is now found to contain an outlier, γ-7, which has significantly lower identity with stargazin. Given that STG-1 and STG-2 can partly substitute for stargazin, it would not be unexpected if additional TARPs, with more limited identity to stargazin, were identified in the mouse genome.

EXPERIMENTAL PROCEDURES

General Methods, Genetics, and Germline Transformation

All *C. elegans* strains were raised under standard conditions at 20°C. Transgenic strains were generated by germ-line transformation using *lin-15(n765ts)* mutants and pJM23 as a transformation marker (Huang et al., 1994). The following plasmids were used to generate transgenic lines: pDM747, *stg-1* genomic; pDM1105, *stg-2* genomic, pWR5, *Pglr-1*::HA::GLR-1(A687T)::GFP; pDM1199, *Pstg-1*::mCherry; pDM649, *Pstg-1*::GFP; pDM1122, *Pstg-2*::mCherry; pPB1, *Pnmr-1*::GFP; pDM1233, *Pstg-2*::STG-1; pDM1244, *Pstg-1*::STG-2; pCSW163, *Pglr-1*::STG-1::mCherry; pPB45, *Pglr-1*::GLR-1::GFP; pYZ96, *sol-1* genomic; pWR6, *Pglr-1*::HA::GLR-1::GFP; pYZ318, *Pmyo-3*::HA::GLR-1::GFP; pYZ146, *Pmyo-3*::SOL-1; pDM796, *Pmyo-3*::STG-1; pDM1158, *Pmyo-3*::STG-2; pDM1041, *Pmyo-3*::GFP::GluR1; and pDM1099, *Pmyo-3*::GFP::GluR1(L507Y). Additional transgenic lines used were *akIs9*, *Pglr-1*::GLR-1(A687T) (Zheng et al., 1999); *akIs58*; *Pglr-1*::HA::GLR-1(A/T)::GFP + *Psol-1*::SOL-1; and *nuIs25*, *Pglr-1*::GLR-1::GFP. The full-length *stg-2* cDNA was isolated by PCR amplification from *C. elegans* first-strand cDNA (GenBank accession number EU019551). Analysis of the STG-2 protein was aided by the ExPASy suite of programs (Gasteiger et al., 2003).

We carried out an F2 synthetic suppressor screen to identify *stg-2*. Transgenic *akIs58; stg-1(ak104)* worms that overexpressed *Pglr-1*::HA::GLR-1(A687T)::GFP and *Psol-1*::SOL-1, were mutagenized with 50 mM of EMS at the L4 larval stage. F2 progeny of the mutagenized worms were screened for suppression of the lurcher phenotype as previously described (Zheng et al., 1999). *stg-2* was cloned using standard genetic mapping techniques and transformation rescue of *akIs58; stg-1(ak104); stg-2(ak134)* worms.

Additional Plasmids

The oocyte expression plasmids used in reconstitution experiments were pDM657, *glr-1*; pDM350, *sol-1*; pDM654, *stg-1*; pDM1116, *stg-2*; p59/2-rat GluR1; and pGEMHE-mouse stargazin.

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Primary Cultures of Dissociated *C. elegans* Muscle Cells

Muscle cells were dissociated from transgenic worms as described (Walker et al., 2006b). Ectopic expression in muscle cells was achieved using the *myo-3* muscle-specific promoter. Expression of *Pmyo-3*::HA::GLR-1::GFP was used as a marker to identify transgenic muscle cells in culture.

Microscopy and Immunolabeling

Immunolabeling in live transgenic worms was achieved as previously described (Zheng et al., 2004). Briefly, anti-HA polyclonal sera (Molecular Probes) was diluted (1:200) in worm injection buffer and injected in the pseudocoelomic space of transgenic worms. Injected worms were allowed to recover for approximately 6 hr before imaging. Wide-field images were acquired using a Zeiss compound microscope with a Roper CoolSnap camera. Confocal images were acquired with Zeiss LSM 510. Quantification of GFP expression and anti-HA staining (Figure 5B) was achieved using Metamorph software. Briefly, a region was drawn around a section of the ventral cord with GFP expression. Metamorph was used to determine the average fluorescence intensity of both GFP and anti-HA staining in the region. To correct for the background signal, a region of the same area located adjacent to the worm was also measured and then subtracted from the fluorescent signal in the ventral cord.

Electrophysiological Studies

Electrophysiological recordings from the AVA interneuron in vivo were made as previously described (Brockie et al., 2001b; Mellem et al., 2002). Rapid perfusion experiments using dissociated muscle cells in culture were performed using previously described protocols (Walker et al., 2006b). The change in open-tip potential was used to measure the rate of solution exchange. Solutions were delivered using a theta tube mounted on the piezoelectric manipulator (Burleigh). Recordings from *Xenopus* oocytes were performed using standard two-electrode voltage clamp techniques (Walker et al., 2006a).

Behavioral Analysis

Nose-touch response, osmotic avoidance, and the average duration of forward movement (Brockie et al., 2001b; Mellem et al., 2002) were performed using published protocols. The standard Student's t test was used to determine statistical significance. Error bars throughout represent the SEM.

SUPPLEMENTAL DATA

The Supplemental Data include one figure and Supplemental Experimental Procedures and can be found with this article online at http://www.neuron.org/cgi/content/full/59/6/997/DC1/.

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CHAPTER 3

SOL-2 AND SOL-1 CUB-DOMAIN PROTEINS FORM A NOVEL EXTRACELLULAR SCAFFOLDING COMPLEX THAT REGULATES SYNAPTIC AMPA RECEPTOR FUNCTION

Abstract

The neurotransmitter glutamate mediates rapid excitatory synaptic transmission by activating ionotropic, or channel-forming, glutamate-gated receptors (iGluRs) (Hollmann et al. 1994; Collingridge et al. 2009). AMPA receptors (AMPARs) are a class of iGluRs that are strongly implicated in learning and memory (Kessels et al. 2009). Modulation of the strength of synaptic communication by glutamate is achieved in part by dynamic changes in functional AMPAR number (Malinow et al. 2002), or modulation of the kinetics of AMPAR gating by their association with auxiliary proteins (Milstein et al. 2008). In *Caenorhabditis elegans*, glutamate-gated current mediated by the GLR-1 AMPAR depends on the transmembrane CUB-domain protein SOL-1, an auxiliary protein that strongly modifies the kinetics of AMPAR function (Zheng et al. 2004; Walker et al. 2006; Walker et al. 2006; Zheng et al. 2006). This finding has been extended by recent studies on the vertebrate proteins Neto1 and Neto2, which demonstrated that modulation of iGluRs by CUB-domain proteins is evolutionarily conserved (Ng et al. 2009; Zhang et al. 2009). However, the SOL-1 proteins in *C. elegans* and the Neto proteins in vertebrates belong to two different classes. Here, we identify SOL-2, a CUB domain protein in *C. elegans* that is homologous to Neto proteins, yet is required for AMPAR function. We find that SOL-2 is required to bind to and stabilize the extracellular domain of SOL-1, forming a protein complex that co-localizes with AMPARs and regulates the kinetics of desensitization. In reconstitution studies, we demonstrate that GLR-1-mediated currents depend on both SOL-1 and SOL-2. In addition, we show that GLR-1 and SOL-2 surface

delivery is independent of SOL-1 trafficking. Currents in *sol-1* mutants can be rescued in a SOL-2 dependent fashion by *in trans* delivery of the soluble extracellular domain of SOL-1. Our results demonstrate that a complex of CUB-domain proteins has a scaffolding role that contributes to the function of synaptic AMPARs.

Introduction

The neurotransmitter glutamate mediates excitatory synaptic transmission by activating multiple classes of tetrameric ionotropic glutamate receptors (iGluRs) (Hollmann et al. 1994). Although many iGluRs are functional when expressed in heterologous cells (Hollmann et al. 1989) , recent studies have revealed that the AMPA class iGluR is associated with many transmembrane proteins, which appear to provide essential auxiliary functions (Tomita 2010). The best studied of these auxiliary subunits are the TARPs (**T**ransmembrane **A**MPAR **R**egulatory **P**rotein) (Milstein et al. 2008), but recently several additional transmembrane proteins have been implicated in AMPAR function (Schwenk et al. 2009; Diaz 2010; von Engelhardt et al. 2010). In *Caenorhabditis elegans*, the GLR-1 AMPAR mediates glutamate-gated current in a subset of interneurons that control movement and the avoidance of noxious stimuli (Hart et al. 1995; Maricq et al. 1995). Genetic and reconstitution studies have demonstrated that a synaptic transmembrane protein complex is required for glutamate-mediated currents and behavior in *C. elegans* (Zheng et al. 2004; Wang et al. 2008). This complex contains GLR-1, SOL-1, a type 1 transmembrane protein containing protein-protein interaction motifs called

CUB-domains (**C**omplement,**U**rchin EGF, **B**MP), and the TARPs STG-1 or STG-2. SOL-1 was shown to regulate the rate of GLR-1 desensitization as well its rate of recovery from desensitization (Walker et al. 2006; Walker et al. 2006; Zheng et al. 2006).

More recently, Neto2 and Neto1 have been identified in mice. These CUB-domain containing transmembrane proteins contribute to signaling mediated by kainate and NMDA (*N*-methyl-D-aspartate) iGluRs, respectively (Ng et al. 2009; Zhang et al. 2009). Whereas SOL-1 contains four predicted protein-protein interaction CUB domains, Neto2 and the closely related Neto1, contains two CUB domains and an LDLa (low-density lipoprotein receptor class A) domain. To date, SOL-1 homologues have not been identified in vertebrates, and Neto homologues have not been identified in invertebrates. In an earlier reconstitution study (Zheng et al. 2006), we found that the extracellular domain of SOL-1 was not required for rescue of behavior and current in transgenic *sol-1* mutants. A prediction of this result was that an additional protein contributes to the GLR-1 receptor complex and that this protein helps recruit SOL-1 to the receptor complex by binding to the soluble extracellular domain of SOL-1 (sSOL-1).

Here, we use a genetic strategy to identify this putative interacting protein and to test the hypothesis that it contributes to AMPAR-mediated synaptic transmission. We identified SOL-2, a CUB-domain protein that is the homologue of the vertebrate Neto proteins. GLR-1 mediated currents were severely diminished in *sol-2* mutants, despite apparently normal surface expression of GLR-1. We found that sSOL-1 rescue of *sol-1* mutants was dependent on SOL-2. Furthermore, we found that *sol-1* mutants were rescued

when sSOL-1 was supplied *in trans*, indicating that the trafficking and synaptic localization of a receptor complex containing TARP, SOL-2 and GLR-1 are not themselves dependent on SOL-1.

Materials and Methods

General Methods, Genetics and Germline Transformation

All *C. elegans* strains were raised under standard laboratory conditions at 20° C. Transgenic strains were generated by injecting pPD97.98, a GFP marker that labels worm coelomocytes (Miyabayashi et al. 1999), or pDM1494, an mCherry marker that labels command interneuron AVA, into desired worms; or in most cases, by injecting pJM23, a rescuing plasmid for worm multi-vulva (Muv) phenotype, into *lin-15(n765ts)* Muv mutants (Huang et al. 1994).

The following plasmids were used to generate transgenic lines: pYZ9, *Pglr-1*::GLR-1(A687T); pWR5, *Pglr-1*::HA::GLR-1(A687T)::GFP; pYZ298, *Pnmr-1*::GFP::sSOL-1; pYZ318, *Pmyo-3*::HA::GLR-1::GFP; pYZ147, *Pmyo-3*::GFP::SOL-1; pYZ327, *Pmyo-3*::GFP::sSOL-1; pDM796, *Pmyo-3*::STG-1; pWR34, *Pmyo-3*::SOL-2; pYZ96, *Psol-1*::SOL-1; pDM1284, *Pglr-1*::mCherry; pDM1196, *Pnmr-1*::mCherry; pDM1755, *Psol-1*::mCherry; pDM1427, *Psol-2*::GFP; pWR37, *Prig-3*::SOL-2::GFP; pDM1556, *Prig-3*::HA::GLR-1::mCherry; pDM1490, *Prig-3*::NMR-2::mCherry; pDM1558, *Psol-2*::GFP::SOL-2;

Additional transgenic lines used were *akIs131*, *Pnmr-1*::GFP::sSOL-1+

Pglr-1::GLR-1(A687T); and *akIs116*, *Pglr-1*::HA::GLR-1(A687T)::GFP. The full-length *sol-2* cDNA was isolated by PCR amplification from a *C. elegans* first-strand cDNA library. The ExPASy suite of programs were used for analysis of the SOL-2 protein (Gasteiger et al. 2003).

A standard F2 genetic suppressor screen was performed to identify *sol-2*. Transgenic *sol-1(ak63); glr-1(ky176); akIs116* worms that overexpressed *Pglr-1*::HA::GLR-1(A687T)::GFP, were mutagenized with 50 mM of EMS at the L4 larval stage. F2 progeny of the mutagenized worms were screened for suppression of the *lurcher* phenotype. *sol-2* was cloned using standard genetic mapping techniques and transformation rescue of the suppressor worms.

Additional Plasmids

The oocyte expression plasmids used in reconstitution experiments were: pDM657, *glr-1*; pDM350, *sol-1*; pDM323, *solube sol-1*; pDM654, *stg-1*; pWR32, *sol-2*.

The BiFC constructs used were: pDM1689, *Prig-3*::VC155::SOL-2; pDM1672, *Prig-3*::VN173::SOL-1; pDM1664, *Prig-3*::VN173::GLR-1; pDM1687, *Pmyo-3*::VN173::sSOL-1; pDM1752, *Prig-3*::VN173::NMR-2.

Primary Cultures of Dissociated *C. elegans* Muscle Cells

Muscle cells were dissociated from transgenic worms as described (Walker et al. 2006). Ectopic expression in muscle cells was achieved using the *myo-3* muscle specific promoter. Expression of *Pmyo-3*::HA::GLR-1::GFP was used as a marker to identify

transgenic muscle cells in culture.

Microscopy

Confocal images were acquired using Nikon Ti-eclipse with 1.49nA x100 objective. Images were scanned by Yokogawa CSU10 and captured by Cascade 1224B EMCCD camera.

Electrophysiological Studies

Electrophysiological recordings from the AVA interneuron *in vivo* were made as previously described (Mellem et al. 2002). Recordings from *Xenopus* oocytes were performed using standard two-electrode voltage clamp techniques.

Behavioral Analysis

Assays, including nose touch response, osmotic avoidance and the average duration of forward and backward movements were performed using published protocols (Mellem et al. 2002). Statistical significance was determined by using the standard Student's t test. Error bars represent the SEM.

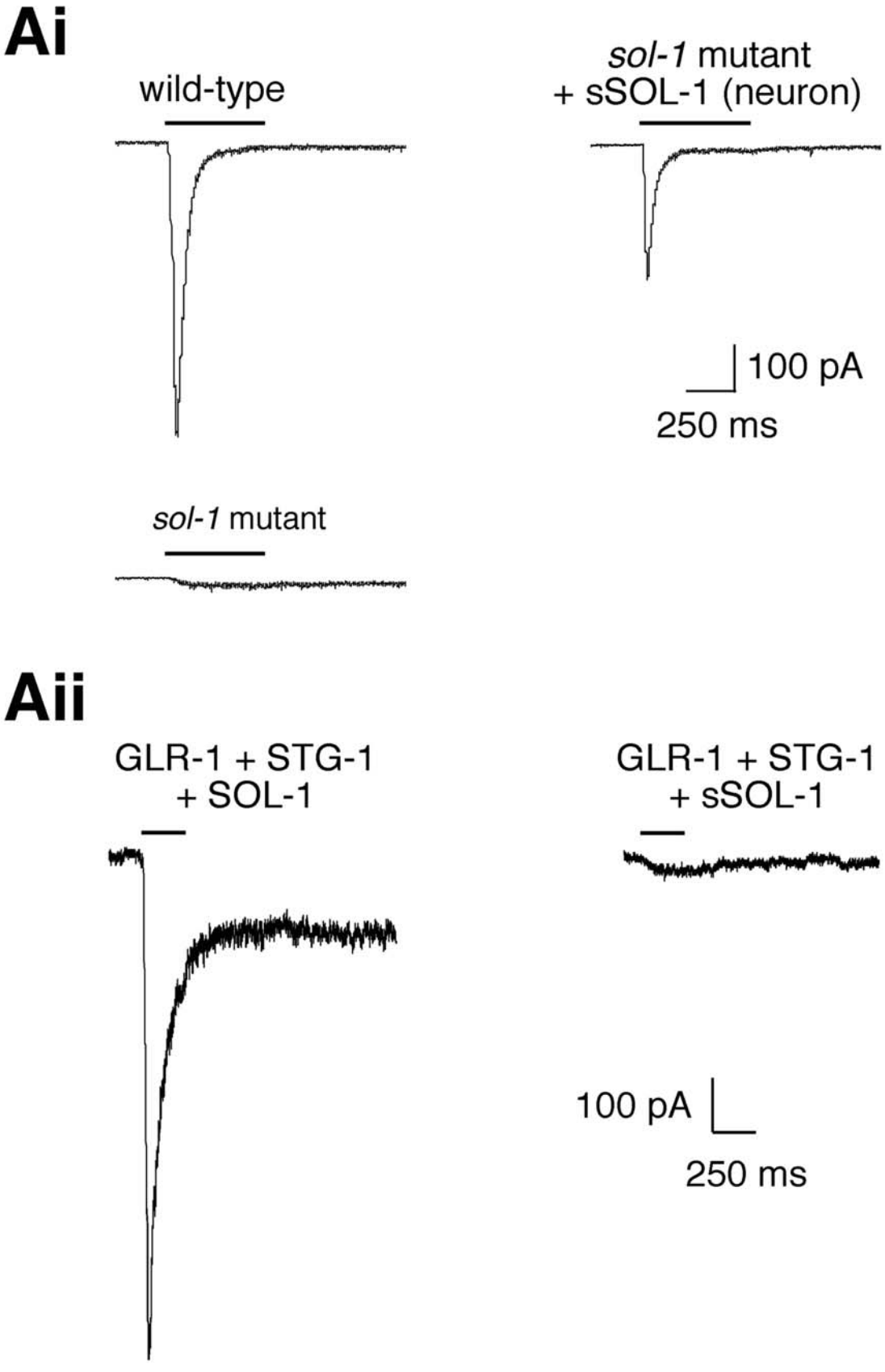
Results

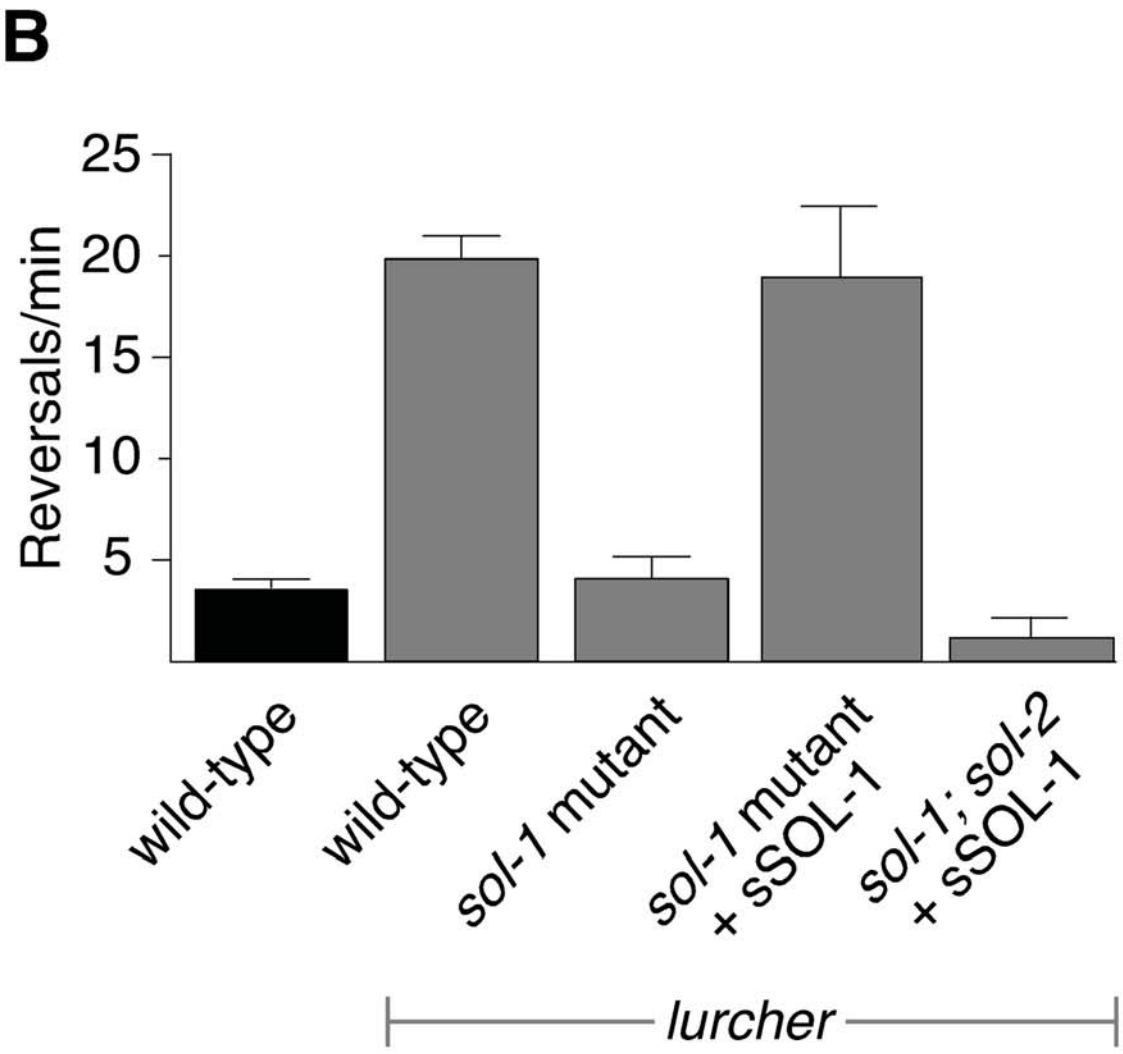
When expressed in neurons of transgenic *sol-1* mutants, sSOL-1 partially rescued the glutamate-gated current (Figure 3.1Ai). This result suggested that the extracellular domain of SOL-1 formed a stable complex with GLR-1 and worm TARPs (STG-1 and STG-2). To test this idea, we asked whether sSOL-1 was sufficient to reconstitute GLR-1

function. Muscle cells in *C. elegans* do not express any known iGluRs, STGs or SOL-1 proteins, and are thus ideal for reconstitution studies. We co-expressed sSOL-1 or SOL-1 along with STG-1 and GLR-1 in the muscle cells of transgenic worms and used patch-clamp electrophysiology to record currents in response to pressure application of glutamate. We recorded large, rapidly-activating inward currents in muscle cells that expressed SOL-1. In contrast, we were unable to record appreciable currents in cells that expressed sSOL-1 (Figure 3.1Aii). These results led us to hypothesize that neurons, but not muscle cells, express a protein that binds to sSOL-1 and contributes to GLR-1 function.

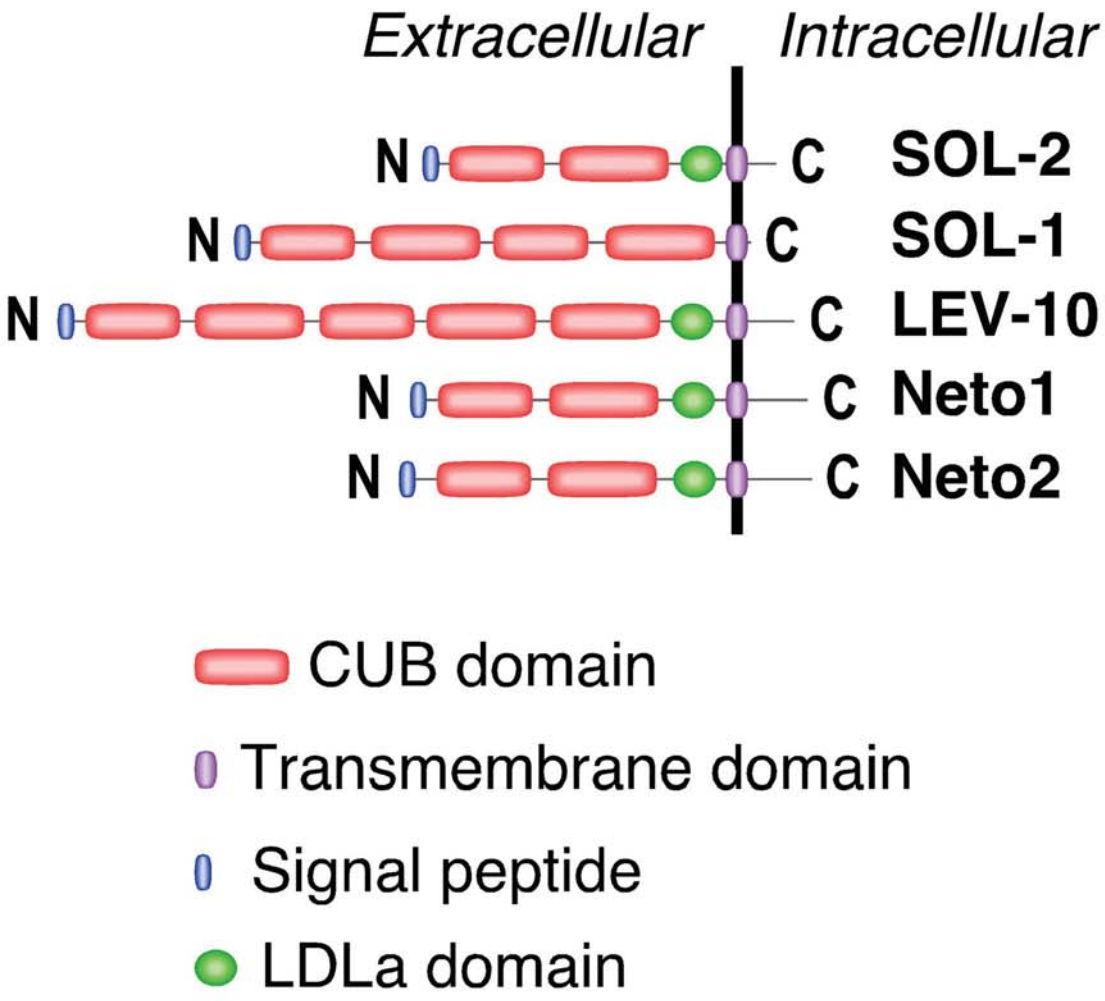
To identify this interacting protein, we turned to a genetic approach. We previously demonstrated that transgenic worms that express GLR-1(A687T), a gain-of-function variant of GLR-1, have a movement defect that we called "*lurching*", which is characterized by frequent reversals in the direction of movement compared to wild-type worms (Zheng et al. 1999). The hyperreversal phenotype of transgenic *lurcher* worms is suppressed in *sol-1* mutants (Figure 3.1B). The sufficiency of sSOL-1 for glutamate-gated currents is also seen in behavior that is dependent on neuronal iGluRs. Thus, transgenic *sol-1*; *lurcher* worms regain their *lurching* behavior if sSOL-1 is expressed in neurons (Figure 3.1B). We screened approximately 2000 genomes for mutations that modified the *lurcher* phenotype. We identified a single mutant that suppressed lurching in both transgenic *lurcher* worms and transgenic *sol-1*; *lurcher* mutants that overexpressed neuronal sSOL-1 (Figure 3.1C). We named this mutant *sol-2*(*ak205*). The *ak205* mutation did not complement *sol-1*, *stg-1* or *stg-2* mutants. We mapped *sol-2* to a small interval on

Figure 3.1 *sol-2* was identified to be required for the neuronal function of soluble SOL-1 and GLR-1.
(Ai) Glutamate-gated currents recorded from AVA neurons in wild-type worms, *sol-1(ak63)* mutants, and *sol-1(ak63)* mutants that expressed soluble SOL-1 (sSOL-1) driven by a neuronal *nmr-1* promoter (*Pnmr-1*::GFP::sSOL-1). Bar indicates pressure application of 1mM glutamate. Cells were held at -60mV.
(Aii) Patch-clamp current records measured in response to pressure application of 1mM glutamate to body wall muscle cells that expressed GLR-1, STG-1 and SOL-1 or soluble SOL-1.
(B) Reversal frequency in wild-type, *lurcher*, *sol-1(ak63); lurcher*, *sol-1(ak63); lurcher* that expressed *Pnmr-1*::GFP::sSOL-1, and *sol-1(ak63); sol-2(ak205); lurcher* that expressed *Pnmr-1*::GFP::sSOL-1.
(C) Schematic representation of the domain structures of the five CUB-domain containing proteins: *C. elegans* SOL-2, SOL-1, LEV-10, and vertebrate Neto1 and Neto2.


Ai
wild-type
sol-1 mutant
+ sSOL-1 (neuron)
100 pA
250 ms
sol-1 mutant
Aii
GLR-1 + STG-1
+ SOL-1
GLR-1 + STG-1
+ sSOL-1
100 pA
250 ms


B
Reversals/min
25
20
15
10
5
wild-type
wild-type
sol-1 mutant
sol-1 mutant + sSOL-1
sol-1; sol-2 + sSOL-1
lurcher

C

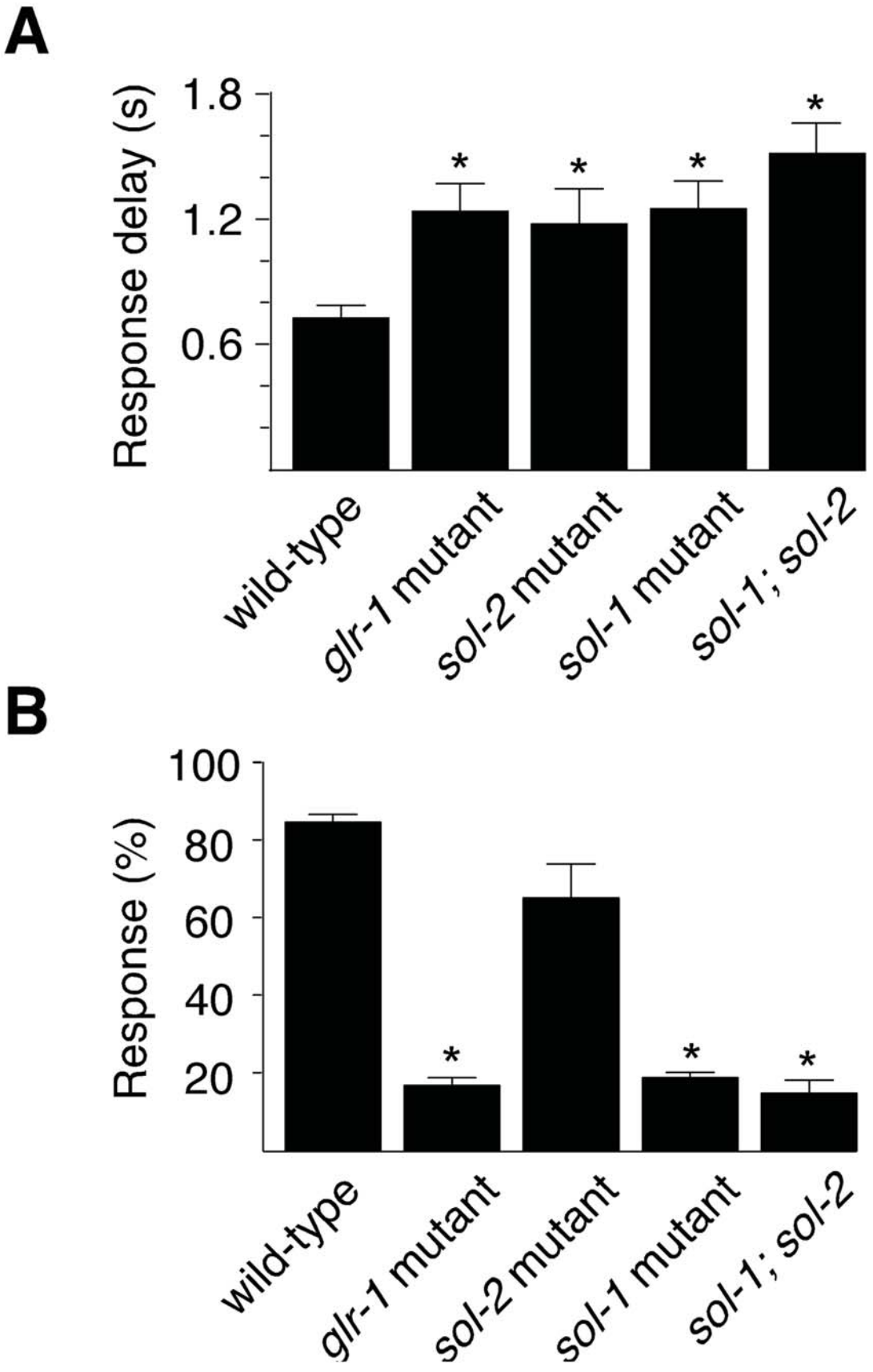

Extracellular
Intracellular
N
C
SOL-2
N
C
SOL-1
N
C
LEV-10
N
C
Neto1
N
C
Neto2
CUB domain
Transmembrane domain
Signal peptide
LDLa domain

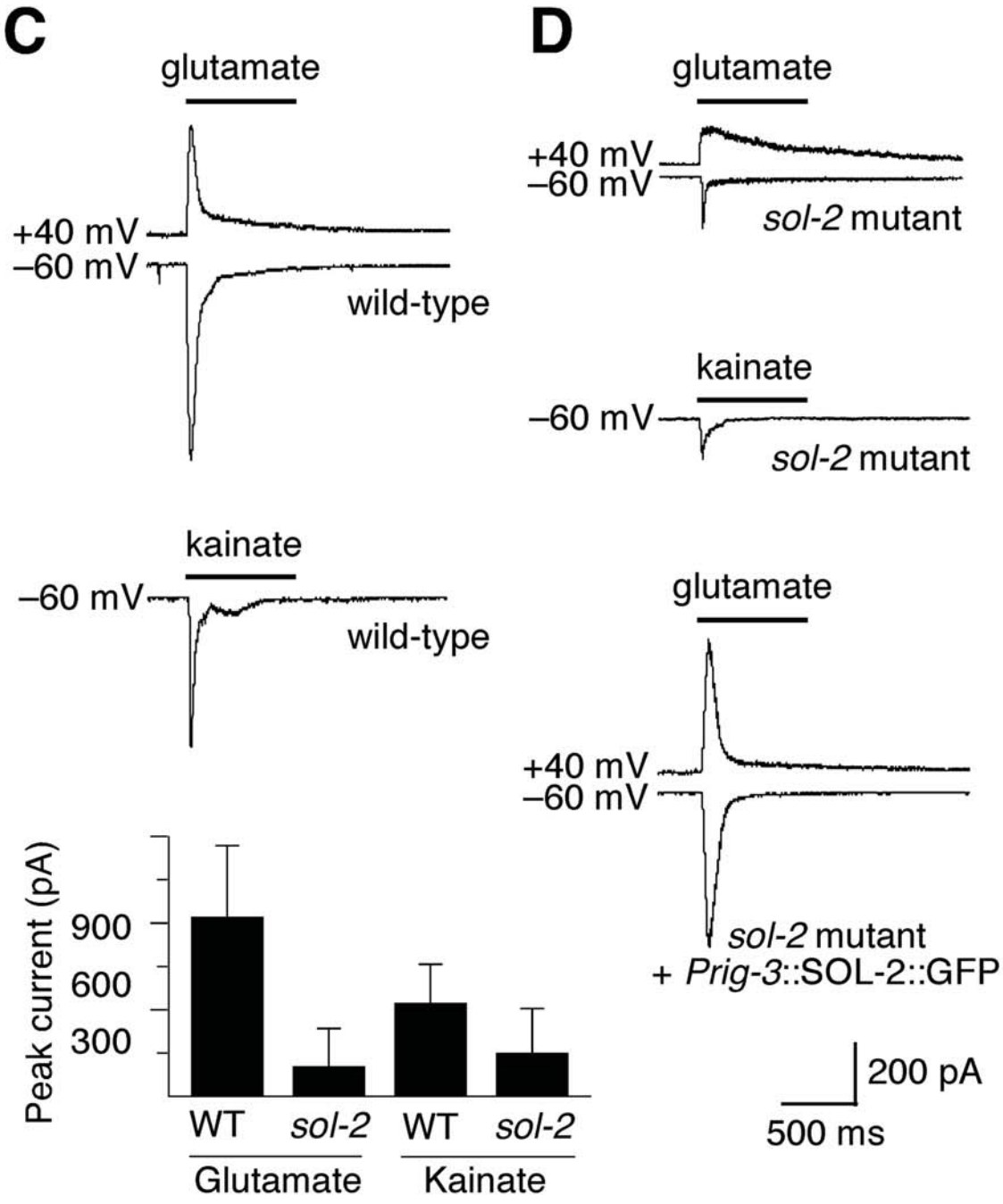
CHR I and using standard cosmid rescue techniques, we identified a mutation in an open reading frame in cosmid K05C4.11. *sol-2* is predicted to encode a 436 amino acid (aa) type 1 transmembrane protein containing two putative CUB domains and an LDLa domain, with strong sequence identity to the vertebrate Neto1 and Neto2 proteins (Figure 3.1C). The *ak205* allele contains an early stop caused by frame shift, suggesting that this mutation is a null.

We studied the behavior of *sol-2* mutants using two standard assays that depend on GLR-1 function. When tested in an osmotic avoidance assay, the *sol-2* mutants were as slow to recoil as *glr-1* or *sol-1* mutants (Figure 3.2A). However, when tested in a nose touch-avoidance assay, *sol-2* mutants were not as impaired as *glr-1* or *sol-1* mutants (Figure 3.2B). In both assays, *sol-1*; *sol-2* double mutants were no more impaired than *sol-1* mutants alone, suggesting that the two gene products do not act in parallel pathways.

The glutamate-gated current in *sol-2* mutants was considerably smaller than in wild-type worms, but still detectable in contrast to the lack of apparent current in *sol-1* mutants (Figure 3.2C, D). Only the GLR-1-mediated current is decreased in *sol-2* mutants; the slower, outwardly rectifying current mediated by NMDA receptors does not appear significantly different than wild-type (Figure 3.2C). Glutamate-gated currents in AVA were restored in transgenic *sol-2* mutants that expressed wild-type SOL-2 specifically in AVA (*Prig-3*::SOL-2::GFP), indicating that SOL-2 can function cell autonomously. However, unlike the case for sSOL-1, we observed no rescue of *sol-2* mutants that expressed a SOL-2 variant lacking the TM domain (data not shown).

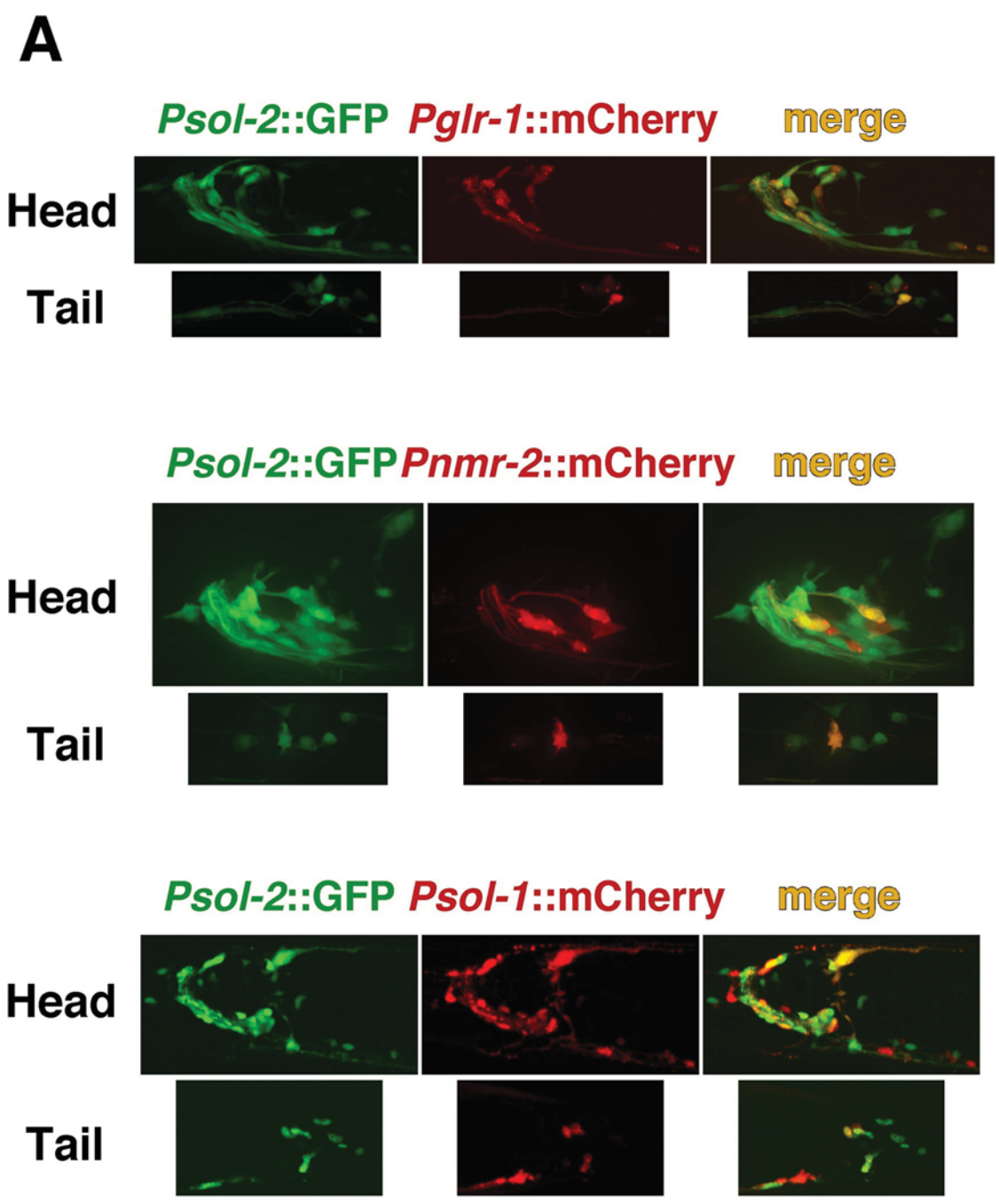
Figure 3.2 The disruption of *sol-2* impairs glutamate-gated currents and worm avoidance behavior mediated by GLR-1 AMPARs.
(A) The delay in the response to hyperosmotic stimuli in wild-type, *glr-1(ky176), sol-2(ak205)*, *sol-1(ak63)* and *sol-1(ak63); sol-2(ak205)*. n=10. *significantly different from wild-type, $p<0.01$.
(B) Percentage of positive nose touch responses in wild-type, *glr-1(ky176)*, *sol-2(ak205)*; *sol-1(ak63)*; and *sol-1(ak63); sol-2(ak205)*. n=10. *significantly different from wild-type, $p<0.01$.
(C and D) Currents measured in response to 1mM glutamate or 100uM kainate in the AVA interneurons of wild-type worms, *sol-2(ak205)* mutants, and *sol-2(ak205)* mutants expressing *Prig-3*::SOL-2::GFP.


A
Response delay (s)
1.8
1.2
0.6
*
*
*
*
wild-type
glr-1 mutant
sol-2 mutant
sol-1 mutant
sol-1; sol-2
B
Response (%)
100
80
60
40
20
*
*
*
wild-type
glr-1 mutant
sol-2 mutant
sol-1 mutant
sol-1; sol-2

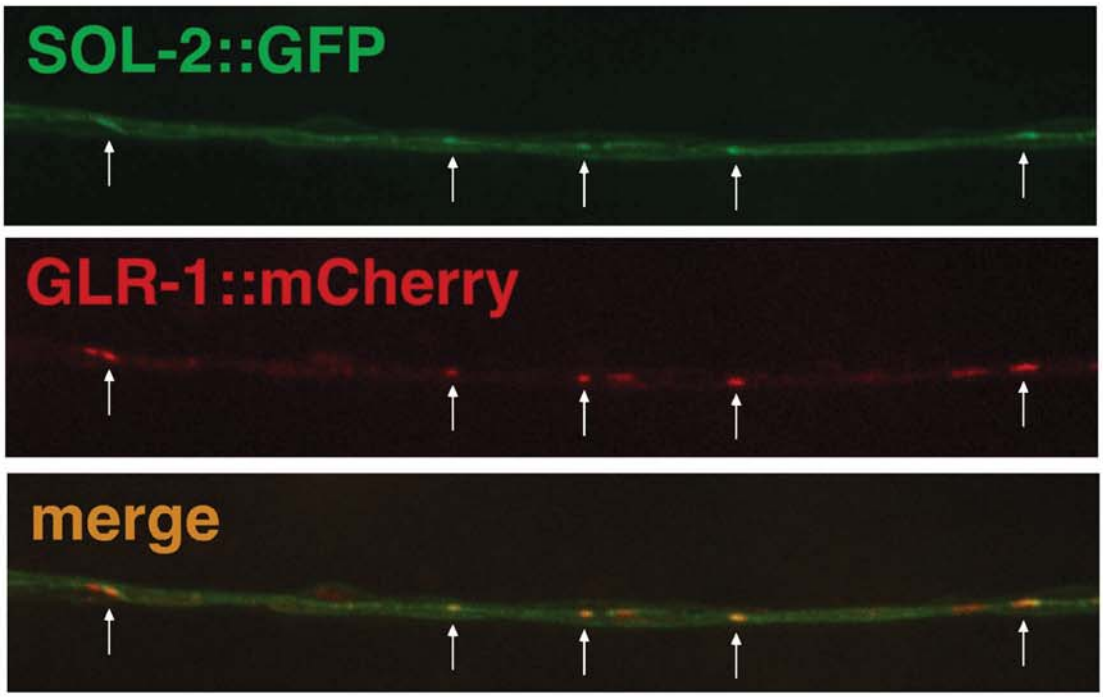

C
glutamate
+40 mV
–60 mV
wild-type
kainate
–60 mV
wild-type
Peak current (pA)
900
600
300
WT
sol-2
WT
sol-2
Glutamate
Kainate
D
glutamate
+40 mV
–60 mV
sol-2 mutant
kainate
–60 mV
sol-2 mutant
glutamate
+40 mV
–60 mV
sol-2 mutant
+ Prig-3::SOL-2::GFP
200 pA
500 ms

We used confocal microscopy to determine the cellular and subcellular distribution of SOL-2. The *sol-2* promoter drives expression of green fluorescent protein (GFP) in many head and tail neurons (Figure 3.3A). These neurons also express the GLR-1 subunit, as well as the SOL-1 protein (Figure.3.3A). In particular, the command interneurons (indicated by *Pnmr-1*::mCherry), which are critical for the control of locomotion, express both GLR-1 and SOL-2 (Figure.3.3A). To determine the subcellular localization of SOL-2, we imaged transgenic worms that expressed a functional full-length SOL-2::GFP that was coexpressed with GLR-1::mCherry in the AVA command interneuron. We found that SOL-2 co-localized with GLR-1, suggesting that SOL-2 might associate with GLR-1, either directly or indirectly (Figure 3.3B). To test whether SOL-2 could associate with components of the GLR-1 receptor complex, we used BiFC (Bimolecular Fluorescence Complementation) (Shyu et al. 2008). We tagged SOL-2 with the C-terminal half of the fluorescent protein Venus (VC155) and GLR-1 with the N-terminal half of Venus (VN173) and expressed these constructs in AVA. We observed punctate fluorescence along the length of the AVA processes when both constructs were co-expressed, but no fluorescence when either construct alone was expressed (Figure 3.3C). Similar results were observed when we co-expressed SOL-2 and SOL-1 BiFC constructs in AVA (Figure 3.3D). Furthermore, the SOL-1 and SOL-2 BiFC signal co-localized with GLR-1::mCherry (Figure 3.3D). These results indicate that SOL-2 is in close proximity to SOL-1 and GLR-1. To address whether SOL-1 and SOL-2 specifically associate with GLR-1 receptors, we co-expressed in AVA the SOL-2 and SOL-1 BiFC constructs along with either

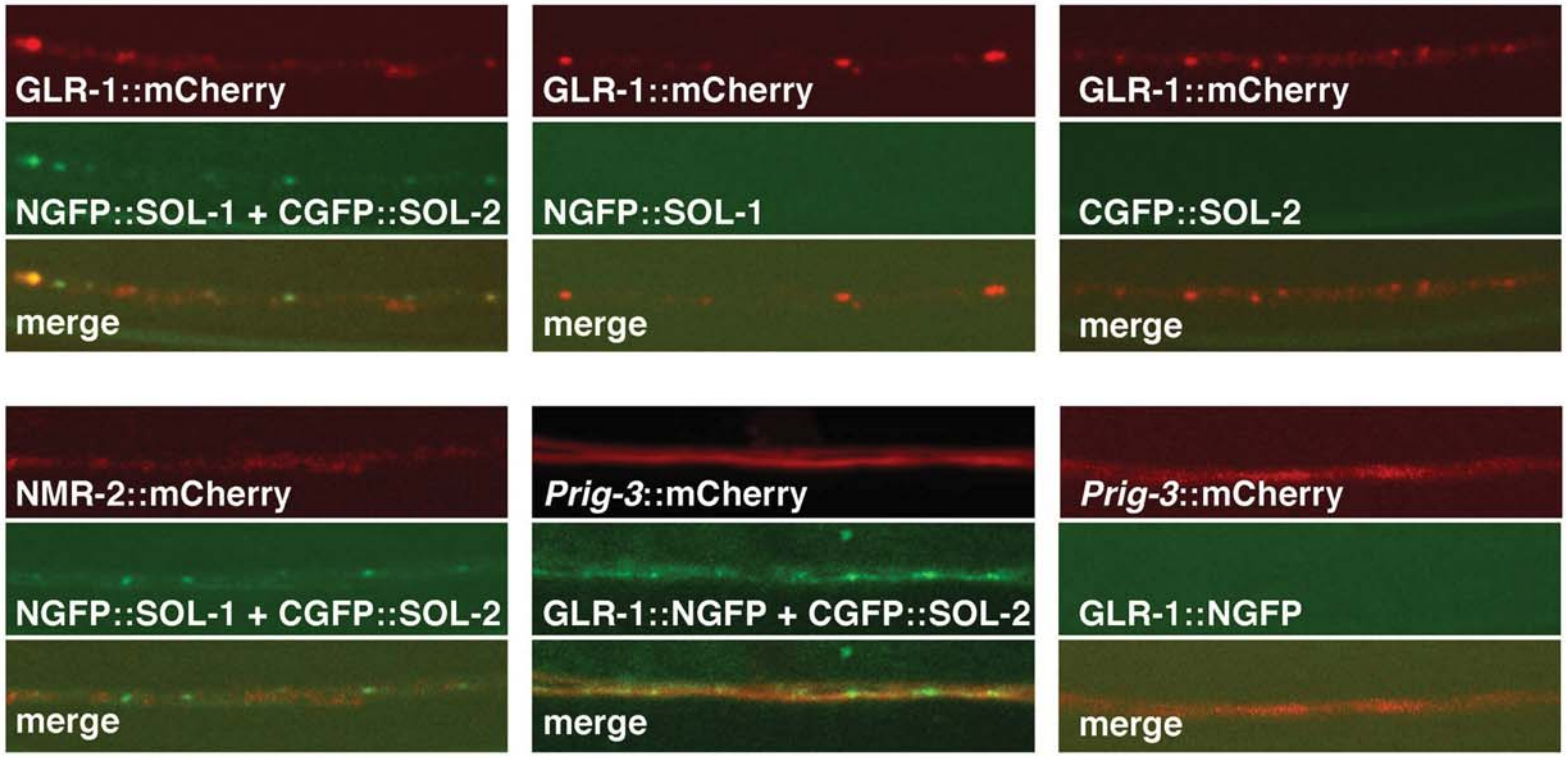
Figure 3.3 SOL-2 co-localizes with SOL-1 and GLR-1.
(A) Confocal images of transgenic worms that expressed *Psol-2*::GFP and *Pglr-1*::mCherry, or *Pnmr-1*::mCherry, or *Psol-1*::mCherry. Head and tail regions only.
(B) Confocal images of the ventral processes in a transgenic worm that expressed functional SOL-2::GFP and GLR-1::mCherry both under the regulation of *rig-3* promoter. Arrow heads indicate puncta thought to represent postsynaptic sites.
(C) (Top) Confocal images of transgenic worms that co-expressed GLR-1::mCherry with VN173::SOL-1, or VC155::SOL-2, or both. (Bottom) Confocal images of transgenic worms that expressed NMR-2::mCherry and VN173::SOL-1+VC155::SOL-2, or *Prig-3*::mCherry and GLR-1::VN173+VC155::SOL-2, or *Prig-3*::mCherry and GLR-1::VN173.


A
Psol-2::GFP
Pglr-1::mCherry
merge
Head
Tail
Psol-2::GFP
Pnmr-2::mCherry
merge
Head
Tail
Psol-2::GFP
Psol-1::mCherry
merge
Head
Tail

B



C

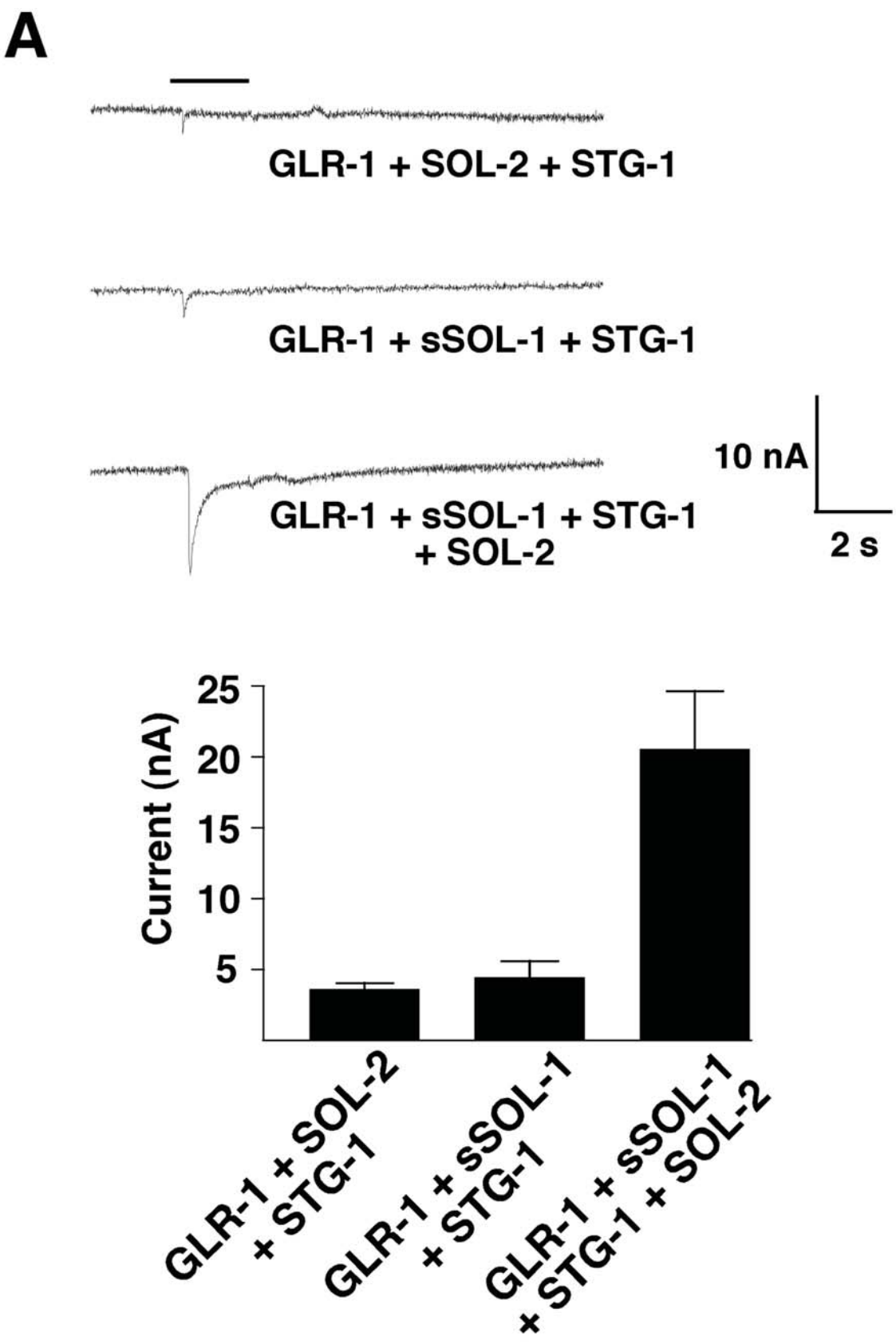

GLR-1::mCherry
NGFP::SOL-1 + CGFP::SOL-2
merge
GLR-1::mCherry
NGFP::SOL-1
merge
GLR-1::mCherry
CGFP::SOL-2
merge
NMR-2::mCherry
NGFP::SOL-1 + CGFP::SOL-2
merge
Prig-3::mCherry
GLR-1::NGFP + CGFP::SOL-2
merge
Prig-3::mCherry
GLR-1::NGFP
merge

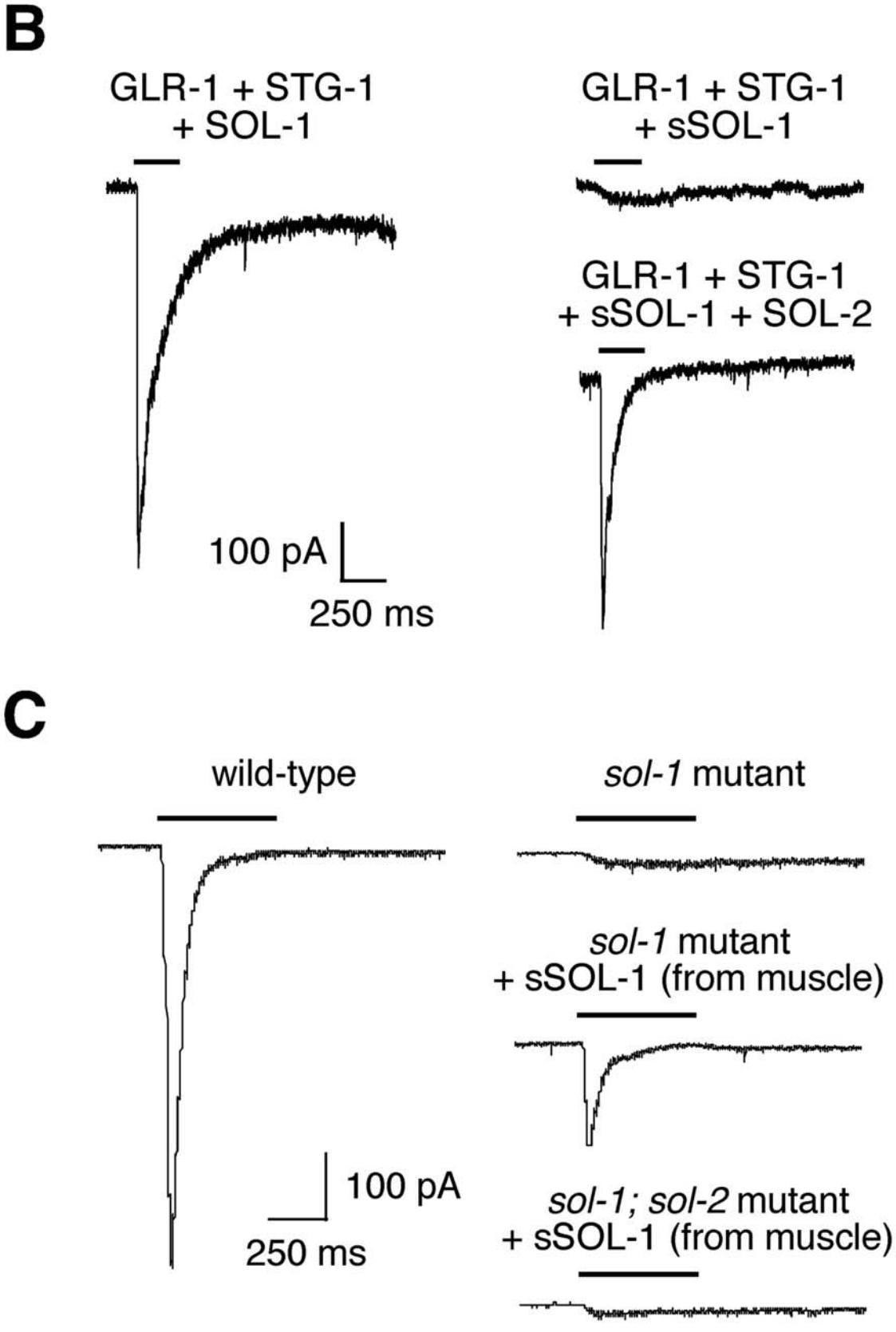
GLR-1::mCherry or NMR-2::mCherry. We observed co-localization of BiFC puncta with GLR-1, but not NMR-2 (Figure 3.3D). These data suggest that SOL-1 and SOL-2 might physically interact and form a complex with GLR-1.

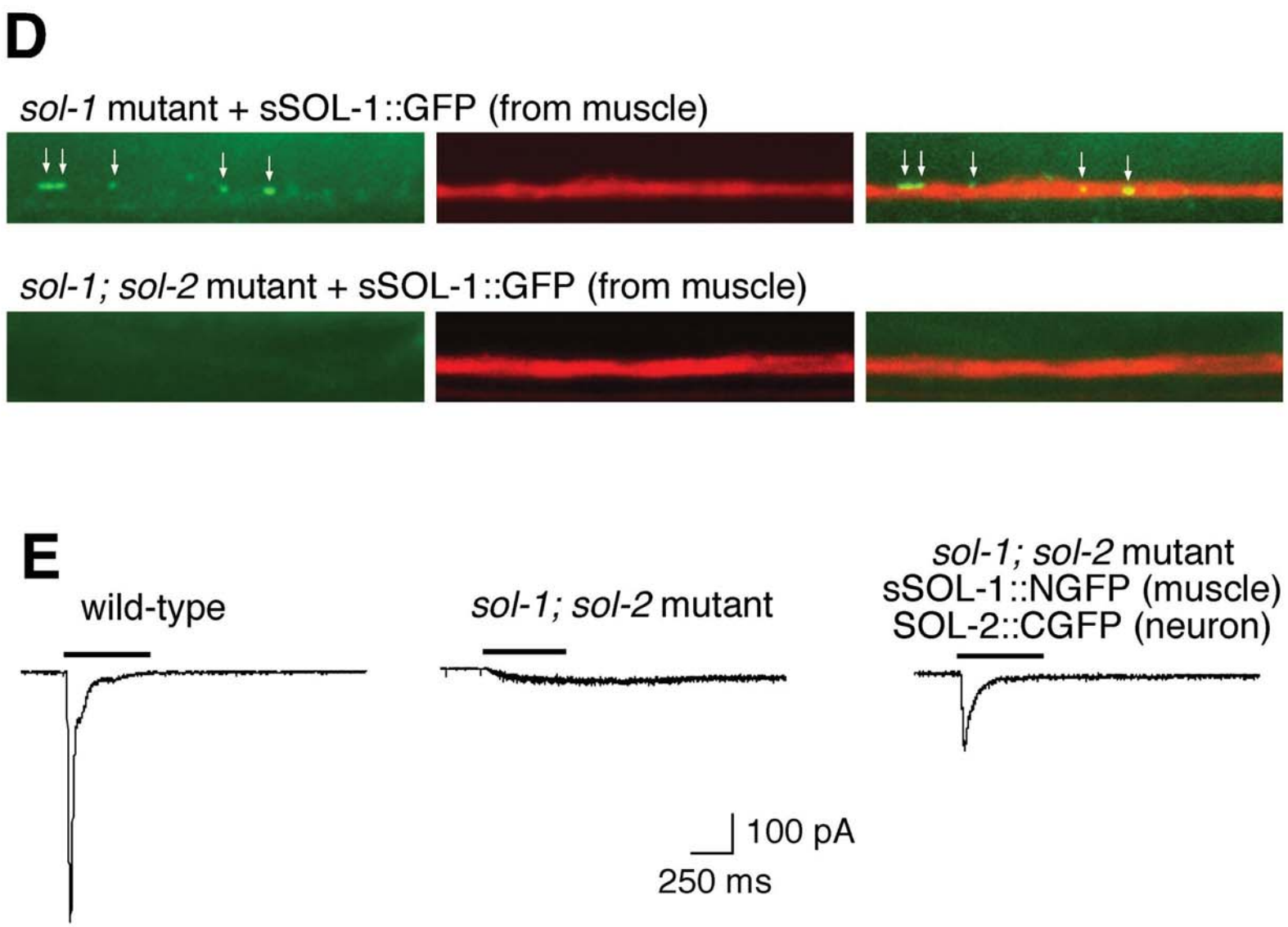
We next asked whether SOL-2 contributed to glutamate-gated currents in reconstitution experiments. We recorded glutamate-gated currents from *Xenopus* oocytes that expressed combinations of *C. elegans* GLR-1, sSOL-1, STG-1 and SOL-2. We did not record appreciable glutamate-gated currents from oocytes that expressed GLR-1, STG-1 and sSOL-1 or GLR-1, STG-1 and SOL-2 (Figure 3.4A). However, we found that co-expression of sSOL-1 and SOL-2 with STG-1 and GLR-1was sufficient to reconstitute glutamate-gated currents (Figure 3.4A). We found similar dependence on SOL-2 when recording glutamate-gated current in AVA of transgenic *sol-1* mutants or *sol-1; sol-2* double mutants where sSOL-1 was expressed in muscles cells and thus supplied *in trans* (Figure 3.4C); or when reconstituting GLR-1 receptor function in transgenic muscle cells (Figure 3.4B).

Our reconstitution experiments also allowed us to address whether SOL-1 has an obligate role in trafficking of GLR-1 or its auxiliary proteins. We found that sSOL-1 can be supplied *in trans* and still rescue glutamate gated currents. Thus, we can reconstitute functional receptors in muscles of transgenic worms when the muscle cells express GLR-1, STG-1 and SOL-2 and the neurons express sSOL-1 (Figure 3.4B). We found the same result in the converse experiment, when the neurons express GLR-1, STG-1 and SOL-2 and the muscles express sSOL-1 (Figure 3.4C). These experiments suggest that muscle

Figure 3.4 *In trans* delivery of soluble SOL-1 restores function in *sol-1* mutants in a SOL-2 dependent manner.
(A) Currents measured in response to 1mM glutamate application in *Xenopus* oocytes that co-expressed GLR-1 and STG-1 with SOL-2, or sSOL-1, or both.
(B) Glutamate-gated currents recorded from worm muscles that co-expressed GLR-1 and STG-1 with SOL-1, or sSOL-1, or sSOL-1+SOL-2.
(C) Current response to 1mM glutamate application in the AVA interneurons of wild-type, *sol-1(ak63)*, transgenic *sol-1(ak63)* that expressed sSOL-1 from muscle (*Pmyo-3*::GFP::sSOL-1), and transgenic *sol-1(ak63)*; *sol-2(ak205)* that also expressed *Pmyo-3*::GFP::sSOL-1.
(D) Confocal images of GFP expression in the ventral cord in transgenic *sol-1(ak63)* mutants and *sol-1(ak63)*; *sol-2(ak205)* double mutants that both expressed *Pmyo-3*::GFP::sSOL-1. Arrow heads indicate puncta thought to represent postsynaptic sites.
(E) Glutamate-gated currents measured in the AVA neurons of wild-type, *sol-1(ak63)*; *sol-2(ak205)*, *sol-1(ak63)*; *sol-2(ak205)* that co-expressed VN173-tagged sSOL-1 secreted from muscle (*Pmyo-3*::VN173::sSOL-1) and neuronal VC155-tagged SOL-2(*Prig-3*::VC155::SOL-2).


A
GLR-1 + SOL-2 + STG-1
GLR-1 + sSOL-1 + STG-1
GLR-1 + sSOL-1 + STG-1
+ SOL-2
10 nA
2 s
Current (nA)
25
20
15
10
5
GLR-1 + SOL-2
+ STG-1
GLR-1 + sSOL-1
+ STG-1
GLR-1 + sSOL-1
+ STG-1 + SOL-2

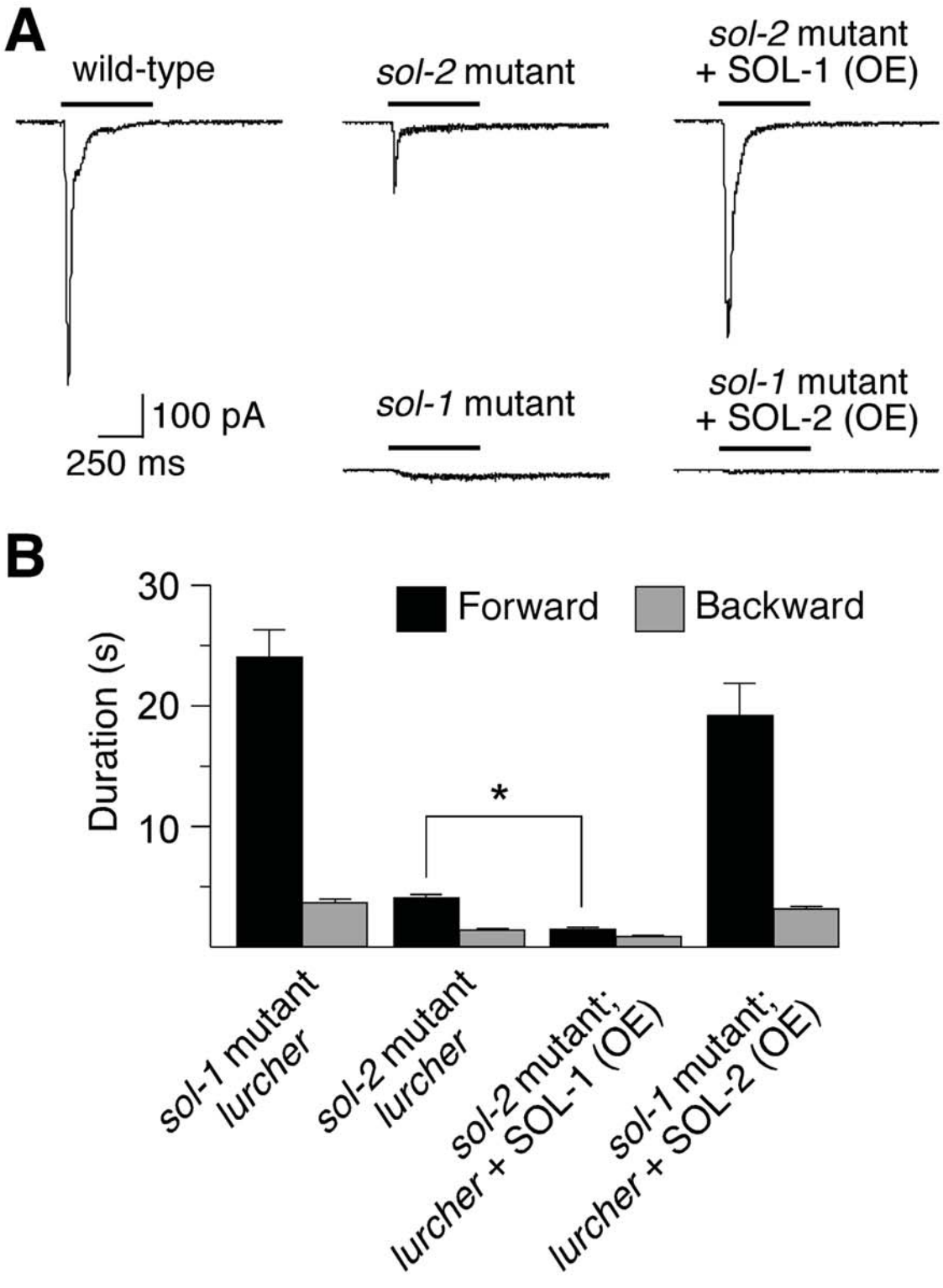

B
GLR-1 + STG-1
+ SOL-1
GLR-1 + STG-1
+ sSOL-1
GLR-1 + STG-1
+ sSOL-1 + SOL-2
100 pA
250 ms
C
wild-type
sol-1 mutant
sol-1 mutant
+ sSOL-1 (from muscle)
100 pA
250 ms
sol-1; sol-2 mutant
+ sSOL-1 (from muscle)


D
sol-1 mutant + sSOL-1::GFP (from muscle)
sol-1; sol-2 mutant + sSOL-1::GFP (from muscle)
E
wild-type
sol-1; sol-2 mutant
sol-1; sol-2 mutant
sSOL-1::NGFP (muscle)
SOL-2::CGFP (neuron)
100 pA
250 ms

secreted sSOL-1 diffuses in the extracellular space and binds to neuronal SOL-2 to reconstitute GLR-1 receptor function. We directly tested this model by examining the fluorescence signal from transgenic *sol-1* mutants that secreted sSOL-1 from muscles. Punctate fluorescence was observed along the ventral cord in *sol-1* mutants, but was eliminated in *sol-1*; *sol-2* double mutants (Figure 3.4D). We also showed that sSOL-1 and SOL-2 BiFC fusion proteins are functional by recording glutamate-gated current in AVA from transgenic *sol-1*; *sol-2* mutants (Figure 3.4E).

Overexpression of SOL-1, STG-1, and GLR-1 in heterologous cells is sufficient to reconstitute receptor function (Walker et al. 2006; Wang et al. 2008). However, no current is apparent in cells that overexpress SOL-2, STG-1, and GLR-1 (Figure 3.4). We have also shown that sSOL-1 function is dependent on SOL-2. These results imply that SOL-2 has an essential scaffolding role that stabilizes SOL-1 association with the GLR-1 receptor complex. We tested this model, by overexpressing SOL-1 in *sol-2* mutants, and overexpressing SOL-2 in *sol-1* mutants. We found current rescue in AVA neurons of *sol-2* mutants that overexpressed SOL-1, but did not find current rescue when SOL-2 was overexpressed in *sol-1* mutants (Figure 3.5A). These results indicate that SOL-2 has a major scaffolding role that organizes the AMPAR signaling complex. A similar result was observed in *lurcher* worms where overexpression of SOL-1 in transgenic *lurcher; sol-2* mutants was sufficient to restore the hyperreversal phenotype, but overexpressing SOL-2 in transgenic *lurcher; sol-1* mutants was not (Figure 3.5B).

Figure 3.5 Overexpression of SOL-1 rescues glutamate-gated currents and behavior in *sol-2* mutants but overexpression of SOL-2 does not rescue currents or behavior in *sol-1* mutants.
(A) Currents measured in response to 1mM glutamate in the AVA interneurons of wild-type, *sol-2(ak205)*, *sol-1(ak63)*, *sol-2(ak205)* that over-expressed SOL-1 under the regulation of *rig-3* promoter, and *sol-1(ak63)* that over-expressed SOL-2 under the same *rig-3* promoter.
(B) Average duration of forward and backward movements for *sol-1(ak63)*; *lurcher*, *sol-2(ak205)*; *lurcher*, *sol-2(ak205); lurcher* that over-expressed genomic SOL-1, and *sol-1(ak63)*; *lurcher* that over-expressed genomic SOL-2. n=10. *significantly different, $p<0.01$.


A
wild-type
sol-2 mutant
sol-2 mutant
+ SOL-1 (OE)
100 pA
250 ms
sol-1 mutant
sol-1 mutant
+ SOL-2 (OE)
B
Forward
Backward
Duration (s)
30
20
10
*
sol-1 mutant
lurcher
sol-2 mutant
lurcher
sol-2 mutant;
lurcher + SOL-1 (OE)
sol-1 mutant;
lurcher + SOL-2 (OE)

Discussion

We identified SOL-2 as a novel auxiliary subunit for GLR-1 AMPARs in *C. elegans* from a genetic screen for suppressors that disrupted normal function of GLR-1and sSOL-1. We showed that in *sol-2* mutants endogenous GLR-1 mediated glutamate-gated currents and behavior are impaired, indicative of SOL-2's contribution to GLR-1 function. We also showed that sSOL-1 functions in a SOL-2 dependent manner. In addition, we provided evidence for direct physical interactions between SOL-2 and SOL-1 or GLR-1, and we further demonstrated that the SOL-1/SOL-2 complex co-localizes with GLR-1, strongly suggestive of the presence of GLR-1 receptor signaling complexes that comprise at least GLR-1, SOL-1 and SOL-2. Finally, we showed that in the presence of GLR-1 and the STG-1 TARP protein, SOL-2, unlike SOL-1, is not sufficient to reconstitute GLR-1 currents, indicating that it does not have a major role in modulating GLR-1 channel properties. Altogether, these results strongly suggest that SOL-2 plays a structural role in recruiting and/or stabilizing SOL-1 and forming functional GLR-1 signaling complexes.

Based on these results we propose a model in which SOL-2 functions as a scaffold that binds to the ectodomain of SOL-1 and stabilizes the association between SOL-1 and GLR-1. In the absence of SOL-2, only a small portion of surface SOL-1 retains association with GLR-1 receptors, greatly diminishing the number of functional signaling complexes. Thus, SOL-2 regulates GLR-1 function by controlling the number of functional iGluRs. In this model, overexpressing SOL-1 overcomes the need for the

stabilizing function of SOL-2. This also implies that the transmembrane domain of SOL-1 maintains weak interactions with GLR-1, even in the absence of SOL-2.

SOL-1 is a four CUB domain containing protein. SOL-2, like its vertebrate homologues Neto1 and Neto2, has two predicted CUB domains and one LDLa domain. CUB domains are known to mediate various protein-protein interactions (Bork et al. 1993). LDLa domain is also likely to be involved in protein-protein interactions. Our model suggests a mechanism for the formation of functional receptor signaling complexes that relies on extracellular protein-protein interactions. This mechanism is likely to be evolutionarily conserved. LEV-10, a *C. elegans* CUB/LDLa protein that resembles SOL-2, has been shown to be required for clustering of acetylcholine receptors at the neuromuscular junction by forming extracellular protein-protein scaffolds (Gally et al. 2004; Gendrel et al. 2009). In addition, Neto1 has been shown to be required for maintaining the abundance of NMDARs in postsynaptic density (Ng et al. 2009), presumably through the same mechanism. Thus, CUB domain proteins are likely important for signaling via both glutamate and acetylcholine and represent a relatively new class of proteins that regulate signaling at synapses.

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CHAPTER 4

SUMMARY AND DISCUSSION

Excitatory neurotransmission in the vertebrate CNS is mostly dependent on ionotropic glutamate receptor (iGluR) subunits that form tetrameric glutamate-gated ion channels. The first iGluR subunit was cloned by reconstituting glutamate-gated current in *Xenopus* oocytes that express the cDNA of GluR1 (GluA1) (Hollmann et al. 1989). Until recently, it was widely accepted that iGluR subunits assembled as tetramers and functioned as "stand-alone" ion channels requiring no additional subunits. However, a decade ago the stargazin/γ2 protein, the founding member of the transmembrane AMPAR regulatory protein (TARP) family, was identified as a necessary component of AMPA receptors. Stargazin was shown to function as an obligate chaperon for AMPARs in *stargazer* mice that have ataxia and absence epilepsy (Chen et al. 2000).

Identifying genes using classical genetic screens in higher animals, such as mice can be a daunting task due to the existence of multiple functionally redundant gene homologues. On the contrary, the simple nematode *C. elegans* is a great model for such identification and studies. In particular, the availability of powerful genetic resources and the well-characterized locomotory behavior in *C. elegans* make it possible and relatively easy to identify specific gene products that play roles in regulating locomotion and thus AMPAR function.

In *C. elegans*, locomotion is controlled, in part, by the GLR-1 AMPAR. Wild-type worms exhibit a stereotypical locomotory pattern with long periods traveling forward interrupted by short-term backward movements. However, in transgenic worms that express a variant of the GLR-1 subunit, GLR-1(A687T), the locomotory pattern is

dramatically changed such that the worms rapidly switch between forward and backward movement (Zheng et al. 1999). GLR-1(A687T) contains the alanine (A) to theronine (T) mutation in the highly conserved SYTANLAAF amino acid motif of TM III (Figure 1.4). This mutation was first identified in the δ2 iGluR subunit of the *lurcher* mouse (Zuo et al. 1997) and transgenic worms that express GLR-1(A687T) are also called "*lurchers"*. In a genetic screen for mutations that modified the *lurcher* phenotype such that they moved normally, Zheng et al. isolated SOL-1, a GLR-1 auxiliary subunit necessary, but not sufficient, for GLR-1 function (Zheng et al. 1999). In Chapter 2 and 3, I further exploited the use of *lurchers* to look for additional auxiliary subunits for GLR-1. From specially designed genetic screens, I identified two TARPs and a CUB-domain protein SOL-2, which also function as novel auxiliary subunits for GLR-1 AMPARs.

***stg-1* and *stg-2* Both Encode TARPs that Modulate GLR-1 AMPAR Kinetics and Regulate GLR-1 Mediated Behavior**

Previous experiments established that reconstitution of GLR-1 function in heterologous systems requires GLR-1, SOL-1 and STG-1. STG-1 encodes a *C. elegans* TARP that is co-expressed with GLR-1 in the nervous system (Walker et al. 2006). To investigate the *in vivo* function of STG-1, *stg-1* deletion alleles were generated. However, the *stg-1* mutants, unlike the *sol-1* mutants, did not significantly suppress the hyper-reversal behavior of transgenic worms that expressed GLR-1(A687T). Furthermore,

glutamate-gated currents were normal in *stg-1* mutants, strongly suggesting that the worm genome encodes additional TARPs that function redundantly with *stg-1*. Candidate gene products were tested for synergistic functions with STG-1, but none of them demonstrated involvement in GLR-1 mediated currents or behavior. Considering this, I resorted to a genetic screen. Using transgenic *stg-1* mutant worms that expressed GLR-1(A687T) and also overexpressed the SOL-1 protein, I screened for synthetic mutations that reduced the hyper-reversal phenotype and isolated *stg-2* mutants, which also encodes a TARP. In *stg-1; stg-2* double mutants, as in *sol-1* mutants, the glutamate-gated current was eliminated, and GLR-1 mediated behaviors such as nose touch and osmotic avoidance, were disrupted as well.

I mapped and cloned the *stg-2* gene which was predicted to encode a 279 amino acid (aa) protein with no significant homology to known proteins (wormbase.org). Further analysis of the *stg-2* coding sequence indicated that it did, in fact, encode a 357 aa TARP homologue. Like STG-1, co-expressing STG-2 with GLR-1 and SOL-1 was sufficient to reconstitute glutamate-gated currents in heterologous cells. These data define STG-1 and STG-2 as the essential group of TARPs that are required for GLR-1 AMPAR function.

The founding member of the TARP family - stargazin was initially found to be required for AMPAR trafficking and surface expression (Chen et al. 2000). To address the possibility that STG-1 and STG-2 have similar roles, I examined the expression levels of surface GLR-1 receptors in wild-type worms, *stg-1* or *stg-2* single mutants, and *stg-1*;

stg-2 double mutants and found no significant difference. This result indicated that GLR-1 surface expression is independent of STG-1 and STG-2, highlighting a functional difference between vertebrate TARPs and their worm homologues.

Behaviorally, *stg-2* mutants mostly resembled *stg-1* mutants, showing no significant defects. Unlike *stg-1* mutants, however, *stg-2* mutants were defective in nose touch avoidance responses. Whereas, the *stg-1*; *stg-2* double mutants were defective in both nose touch and osmotic avoidance responses. This implies that STG-1 and STG-2 could have distinct expression pattern or have differential functions. To address this, first, I performed confocal microscopic imaging to show the cellular expression of STG-1 and STG-2. In large they have an overlapping expression pattern, but are differentially expressed in some neurons. Notably, STG-2, but not STG-1, is expressed in the AVA neuron, one of the command interneurons that express GLR-1 and regulate worm avoidance responses. Furthermore, we examined glutamate-gated currents in *stg-2* mutants and compared these to current measured in *stg-1* and *glr-1* mutants. Although either STG-1 or STG-2 along with SOL-1 and GLR-1 is sufficient to reconstitute glutamate-gated currents in heterologous cells, our rapid perfusion electrophysiological experiments demonstrated that STG-1 and STG-2 differentially modify the kinetics of GLR-1 AMPARs. In detail, STG-1 gives incompletely desensitized glutamate-gated currents, whereas in the presence of STG-2, receptors completely and rapidly desensitize. When both STG-1 and STG-2 are co-expressed with GLR-1 and SOL-1, the peak glutamate-gated current is 10-fold bigger than when co-expression of either STG-1 or

STG-2 alone. Taken together, these data suggest that STG-1 and STG-2 function to fine tune GLR-1 mediated glutamate-gated current to regulate specific worm behaviors.

Both STG-1 and STG-2 have low sequence identity with vertebrate TARPs. To address whether the function of these proteins is evolutionarily conserved, we co-expressed either STG-1 or STG-2, or both STG-1 and STG-2 with vertebrate GluR1 (GluA1) in heterologous cells and recorded glutamate-gated currents. We found that either STG-1 or STG-2 is competent to promote GluR1 mediated currents whereas the surface expression of GluR1 is independent of either one.

Together, the comprehensive genetic and electrophysiological studies point to a fundamental evolutionarily conserved role for TARPs in regulating AMPAR function and behavior.

***sol-2* Encodes a CUB-domain Protein that Organizes and Stabilizes GLR-1 Receptor Signaling Complex**

Previously, we had shown that a soluble version of SOL-1 that lacked the transmembrane domain (sSOL-1) was functional and sufficient for GLR-1 mediated behavior and current in the presence of at least one STG protein. However, co-expressing GLR-1, STG-1 and sSOL-1 was not sufficient to reconstitute glutamate-gated current in *Xenopus* oocytes. These results lead to the hypothesis that a yet to be identified protein is co-expressed in the nervous system with SOL-1 and this proteins is necessary for sSOL-1

to function.

Since sSOL-1 also restores the function of the GLR-1(A687T) *lurcher* receptor in *sol-1* mutants, I used transgenic *sol-1* mutants that expressed GLR-1(A687T) and sSOL-1 in a genetic suppressor screen. These worms show the same hyper-reversal phenotype of *lurcher* worms. I mutagenized these worms and identified mutants with a reduced reversal frequency. I cloned the mutated gene, which we called *sol-2*, and showed that it encoded a type I transmembrane protein that is homologous to the vertebrate CUB-domain proteins Neto1 and Neto2. Interestingly, Neto1 and Neto2 were reported to be auxiliary proteins for NMDARs and kainate receptors, respectively. Given that SOL-1, an obligate auxiliary subunit for GLR-1, is also a CUB-domain protein but with different domain arrangement, it is possible that CUB-domain proteins evolved to both conserved and divergent functional roles.

To further characterize *sol-2* mutants, we examined endogenous GLR-1 mediated behavior and showed that *sol-2* mutants were defective in osmotic avoidance behavior. Nose touch avoidance behavior was only slightly impaired. Furthermore, the glutamate-gated current in *sol-2* mutants was significantly decreased compared to wild-type. Together, these data suggest that SOL-2 is required for endogenous GLR-1 function. The observation that sSOL-1 only functions in the presence of SOL-2 suggests the possibility that SOL-2 may stabilize SOL-1 at the synapse.

To address the possibility that SOL-2 interacts directly with SOL-1 and GLR-1, we examined the cellular and subcellular expression pattern of SOL-2. We found that

SOL-2 is co-expressed with SOL-1 and GLR-1 in many neurons, especially in a subset of command interneurons that mediate locomotion and specific avoidance responses. When expressed in the command interneuron AVA, SOL-2 co-localizes with GLR-1 along the length of the AVA processes where synapses form. In addition, our BiFC experiments showed that SOL-2 directly interacts with SOL-1 as well as GLR-1. In particular, we showed that the SOL-1/SOL-2 BiFC signal co-localizes with GLR-1, but not NMR-2, suggesting that SOL-2 specifically forms complexes with AMPARs and not NMDARs.

To further test the model that SOL-2 binds to SOL-1 and is required for GLR-1 signaling, we co-expressed GLR-1, STG-1 and sSOL-1 either with or without SOL-2 in both *Xenopus* oocytes and transgenic *C. elegans* muscle cells and recorded glutamate-gated currents. We observed significant increases in glutamate-gated currents when SOL-2 was also expressed, indicating that SOL-2 is required for sSOL-1 function. Additionally, we also demonstrated that sSOL-1 tagged with GFP and expressed in muscle cells of transgenic worms rescued glutamate-gated GLR-1 currents in the AVA neuron. The sSOL-1::GFP from muscle also exhibited a punctate expression pattern in AVA of transgenic *sol-1* mutants, but not in transgenic *sol-1; sol-2* double mutants providing further evidence that SOL-2 is necessary for sSOL-1 function in the receptor complex.

Finally, to test if SOL-2 is sufficient to reconstitute GLR-1 function in heterologous cells, we overexpressed SOL-2, STG-1 and GLR-1 in *Xenopus* oocytes. Unlike SOL-1, overexpression of SOL-2 is not sufficient to generate appreciable

glutamate-gated currents. We further showed that overexpressing SOL-1 in transgenic *sol-2* mutants was able to restore glutamate-gated currents, but overexpressing SOL-2 in transgenic *sol-1* mutants was not. All these data indicate that SOL-2 functions as a scaffold and stabilizes SOL-1 association with the GLR-1 receptor complex.

Glutamate Receptor Complex

The identification of the TARPs, STG-1 and STG-2, and the CUB-domain proteins, SOL-1 and SOL-2, in *C. elegans* allowed us to describe a model of the glutamate receptor complex formed by the physically interacting GLR-1 and its auxiliary proteins (Figure 4.1). In this model, GLR-1 iGluR subunits form glutamate-gated ion channels; SOL-1 and STG proteins alter the channel properties of the receptor, including gating speed and desensitization rate; whereas SOL-2 has a scaffolding role that stabilizes the association between SOL-1 and GLR-1 complex.

Structurally, all auxiliary subunits may interact with the receptor directly or indirectly. We have shown that SOL-1 and GLR-1 can be co-immunoprecipitated (Zheng et al. 2004); and SOL-2 interacts with both GLR-1 and SOL-1 in close proximity (Chapter 3). We also showed co-localized synaptic expression of GLR-1with SOL-1 (Zheng et al. 2004), STG-1 (Wang et al. 2008) and SOL-2 (Chapter 3) along neuronal processes at synapses. This is not a complete list of interactions between these transmembrane proteins, but provides solid evidence that auxiliary proteins and receptors form a functional complex. However, the interaction interfaces remain unclear. It is

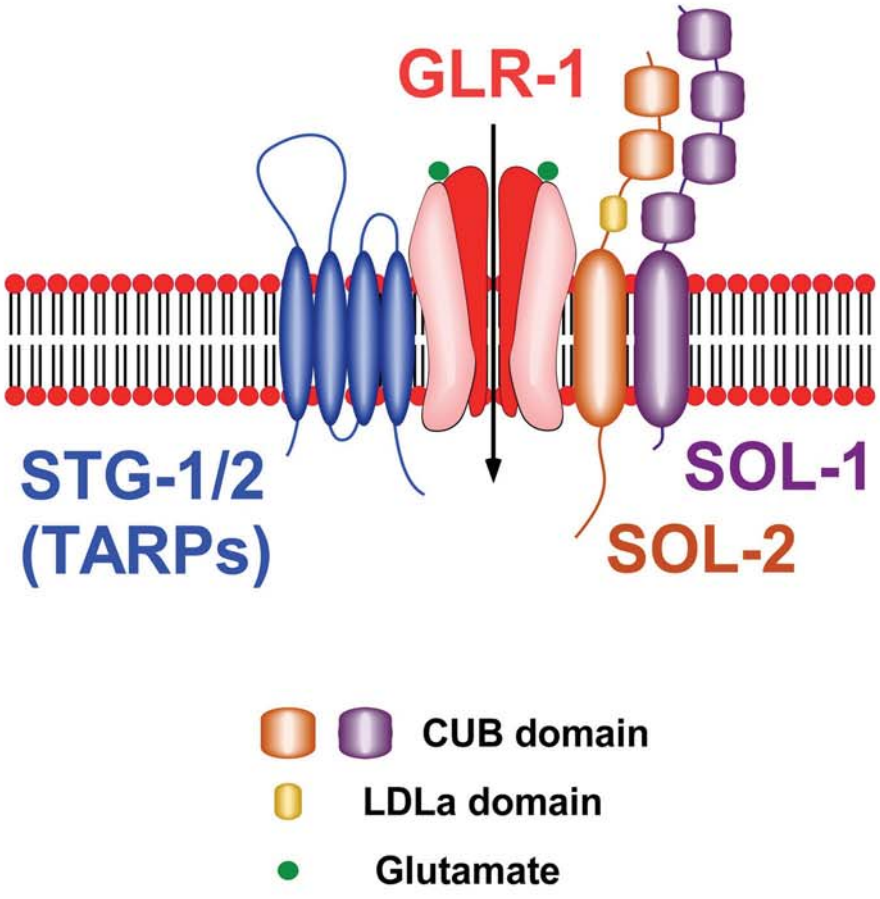

Figure 4.1 A model for GLR-1 glutamate receptor complex. GLR-1 subunits form tetrameric glutamate-gated ion channels. Tetraspanning TARPs, STG-1 and STG-2, and type I transmembrane CUB-domain protein SOL-1 modulate channel properties. Another CUB-domain protein SOL-2 mediates SOL-1 association with the GLR-1 receptor complex.

necessary to determine the atomic structure of the complex to reveal the precise mechanism of the interactions between components of the complex.

We have demonstrated specific functional roles that each auxiliary subunit plays in regulating GLR-1 receptor function. SOL-1 and STG proteins differentially modulate the kinetics of GLR-1 receptors (Walker et al. 2006; Zheng et al. 2006; Wang et al. 2008). These functional differences suggest that the interactions between receptor and each one of the auxiliary subunits have unique mechanisms. Above all, GLR-1, SOL-1 and at least one of the STGs are sufficient to reconstitute GLR-1 function in heterologous cells indicating the functional dependence of receptors upon SOL-1 and the STGs (Walker et al. 2006). SOL-2, however, appears not to be involved in regulating the channel properties. Presumably, by interacting with the ectodomain of SOL-1, SOL-2 stabilizes the interaction between SOL-1 and GLR-1, thereby controls the number of functional receptor complexes.

One factor that would allow us to better understand the composition of the receptor complex would be to determine the stoichiometry between GLR-1 and its auxiliary subunits. In vertebrate, TARPs are found to be associated with AMPARs at variable stoichiometry (4/2/0) (Shi et al. 2009). However, limited by reagents and techniques available in *C. elegans*, this has not been studied for GLR-1 and its auxiliary subunits.

On the other hand, this model only provides a static view of surface GLR-1 receptor complex. It will be intriguing to investigate the upstream processes, such as the

assembly and intracellular trafficking of receptor complexes. Vertebrate AMPARs are likely assembled with TARPs at ER and trafficked together (Vandenberghe et al. 2005). *C. elegans* TARPs, however, do not have this chaperon function and the molecular machinery required for GLR-1 assembly and transport remains elusive.

The dynamic regulation of surface AMPARs that underlies synaptic plasticity is attracting intensive studies world-wide. Profound studies in the involvement of auxiliary proteins in the dynamics of surface GLR-1 turn-over will further our understanding in the regulatory mechanism of synaptic strength.

Concluding Remarks

A fundamental question in neuroscience is how a functional synapse is built, maintained and regulated. Only recently have we been able to identify auxiliary subunits for ionotropic glutamate receptors that mediate the majority of excitatory synaptic transmission in our body. My studies described in this dissertation identified proteins belonging to two families of auxiliary subunits that are essential for the AMPA-class of glutamate receptors. These discoveries provided insight into the molecular mechanisms that regulate AMPAR function and mediate specific behaviors. Further analysis toward these evolutionarily conserved proteins will extend our understanding of how auxiliary proteins contribute to the assembly, localization and turn-over of the receptor signaling complex, the modulation of receptor conformational changes and channel properties, and the interaction with other synaptic proteins.

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